

ASSAY METHOD FOR GROUP TRANSFER REACTIONS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 60/443,746 filed January 30, 2003, which is incorporated by reference here in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

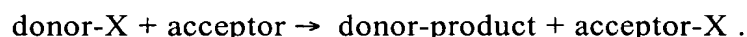
[0002] Not Applicable

TECHNICAL FIELD

[0003] The present invention relates to group transfer reaction methodologies. The invention provides methods for the detection and quantification of donor-products and the catalytic activities generating the donor-products in group transfer reactions. The invention also provides methods for high throughput screening of acceptor substrates, inhibitors, or activators of enzymes catalyzing group transfer reactions. The invention further provides immunoassays, antibodies and related kits for practicing the invention.

BACKGROUND OF THE INVENTION

[0004] There are many important biological reactions where the substrates are modified by chemical groups that are donated by other substrates, known as activated donor molecules. These biological reactions are broadly recognized as “group transfer reactions” and have the general reaction:



Typically, donor-X, the activated donor molecule, is a nucleotide attached to a covalent adduct. The donor-X is activated by formation of a phosphoester bond in the nucleotide donor. Also the acceptor substrates can include small molecules or macromolecules such as proteins or nucleic acids. Products of this reaction are the modified acceptor, acceptor-X and the donor-product molecule.

[0005] There are many enzymes that catalyze group transfer reactions such as for example kinases, which use ATP to donate a phosphate group; sulfotransferases (SULTs), which use phosphoadenosine-phosphosulfate (PAPS) to donate a sulfonate group; UDP-glucuronosyltransferases (UGTs), which use UDP-glucuronic acid to transfer a glucuronic acid group; methyltransferases, which use S-adenosyltransferase to donate a methyl group; acetyl transferase, which use acetyl coenzymeA to donate an acetyl group; and ADP-

ribosyltransferases, which use nicotinamide adenine dinucleotide (NAD) to donate an ADP-ribose group. Thus, many of the enzymes catalyzing group transfer reactions are of interest to pharmaceutical companies.

[0006] Automated high throughput screening (HTS) assays are the paradigm for identifying interactions of potential drug molecules with proteins in a drug discovery setting, and this format requires simple, robust molecular assays, preferably with a fluorescent or chemiluminescent readout. The most suitable format for HTS is a homogenous assay, (e.g., “single addition” or “mix and read” assay), which does not require any manipulation after the reaction is initiated and the assay signal can be monitored continuously. Despite their importance from a drug discovery perspective, the incorporation of group transfer enzymes into pharmaceutical HTS programs is being slowed or prevented for a number of reasons: a) homogenous assay methods are not currently available, b) the detection methods used place serious limits on the utility of the assay, and c) the non-generic nature of the assay requires the development of many specific detection reagents to test diverse acceptor substrates.

[0007] The approach currently used to identify sulfotransferase substrates or inhibitors requires the use of radioactivity and involves cumbersome post-reaction separation steps, such as precipitation or chromatography. For instance, ^{35}S -PAPS is used in a sulfotransferase reaction and the labeled product is quantified by scintillation counting after selective precipitation of unreacted ^{35}S -PAPS (Foldes, A. and Meek, J. L., *Biochim Biophys Acta*, 1973, 327:365-74). This approach is not desirable in a high throughput screening (HTS) format because of the high radiation disposal costs and because the incorporation of separation steps complicates the automation process. Other SULT assays have been developed using colorimetric and fluorescent means, but they are dependent on the use of a specific acceptor substrate for detection, so their use is limited to a single SULT isoform, and they cannot be used to screen for diverse substrates (Burkart, M. D. and Wong, C. H., *Anal Biochem*, 1999, 274:131-7; Frame, L. T., et al., *Drug Metab Dispos*, 2000, 28:1063-8). As a result, SULT interaction studies are currently not included during the preclinical development of drugs. Also UGTs are currently assayed using radiolabeled donor molecules and require post-reaction separation steps such as thin layer chromatography (TLC) or high pressure liquid chromatography (HPLC) which seriously hampers preclinical HTS programs (Ethell, B. T., et al., *Anal Biochem*, 1998, 255:142-7). Likewise, traditionally, kinases have been assayed by filter capture or precipitation of radiolabeled polypeptide substrates produced using ^{32}P -ATP or ^{33}P -ATP as donor. However, since this method requires a separation step such as

filtering or centrifugation, it cannot be easily adapted to an automated HTS format. Surface proximity assays (SPA) allow radioassays in a multiwell format with no separation (Mallari, R., et al., J Biomol Screen, 2003, 8:198-204) but their use by pharmaceutical companies is declining because of the disposal and regulatory costs of handling radioisotopes.

[0008] Because of the high level of interest in developing kinase inhibitor drugs, there has been a great deal of effort, scientists to develop improved assay methods for this enzyme family. Homogenous assay methods have been developed, in which highly specific reagents are used to detect the reaction products in the presence of the other components of the reaction using a light-based readout, such as fluorescence or chemiluminescence. The most common homogenous approach used for kinase assays is immunodetection of phosphopeptide products exhibiting different fluorescence properties (Zaman, G. J., et al., Comb Chem High Throughput Screen, 2003, 6:313-20). In this method, phosphorylation of substrate peptide leads to displacement of a fluorescent phosphopeptide tracer from anti-phosphopeptide and causes a change in its fluorescence properties. This basic approach has been adapted to several different readout modes used for competitive immunoassays including Fluorescence polarization (FP) (Parker, G. J., et al., J Biomol Screen, 2000, 5:77-88); time resolved fluorescence (Xu, K., et al., J Biochem Mol Biol, 2003, 36:421-5); fluorescence lifetime discrimination (Fowler, A., et al., Anal Biochem, 2002, 308:223-31); and chemiluminescence (Eglen, R. M. and Singh, R., Comb Chem High Throughput Screen, 2003, 6:381-7).

[0009] The shortcoming with this approach is the requirement for phosphopeptide-specific antibodies. Though generic phosphotyrosine antibodies are common, phosphoserine and phosphothreonine antibodies are notoriously difficult to produce and only recognize phospho-serine or -threonine in the context of specific flanking amino acids (Eglen and Singh, 2003). There are over 400 kinases in humans and their specificity for phosphorylation sites vary widely. Thus, different antibodies are needed for assaying diverse kinases or profiling acceptor substrates. This greatly complicates the incorporation of new kinases into HTS, especially if their substrate specificity is not well defined. It also creates analysis problems in comparing data among kinases with different substrate selectivities, because the output of the assay depends on the particular antibody(Ab)-phosphopeptide pair used. Although efforts to develop generic phospho-serine antibodies and identify more generic kinase substrates continue (Sills, M. A., et al., J Biomol Screen, 2002, 7:191-214), research in this direction has not been very successful to date.

[00010] A number of alternative approaches have been developed to circumvent the problem of context-specific Ab-phosphopeptide interactions, including use of metal complexes to bind phosphopeptides (Scott, J. E. and Carpenter, J. W., *Anal Biochem*, 2003, 316:82-91) and the use of modified ATP analogs that allow covalent tagging of phosphopeptide products (Allison Miller-Wing, E. G., Barbara Armstrong, Lindsey Yeats, Ram Bhatt, Frank Gonzales, and Steven Gessert., SBS 9th Annual Conference and Exhibition, Portland, OR., 2003). Chemical phosphate binding reagents suffer from background binding to nucleotide phosphates, requiring the use of very low, non-physiological levels of ATP and limiting assay flexibility. Modified nucleotides do not provide a generic format because the ability to use the ATP analogs as donors varies among kinases, requiring the development of a number of different analogs. Also competition of inhibitors with the modified nucleotides at the kinase ATP binding site – the most frequent site for kinase inhibitor binding - does not reflect the physiological situation. Differences in protease sensitivity caused by peptide phosphorylation have also been exploited in developing fluorescence based kinase assays (Kupcho, K., et al., *Anal Biochem*, 2003, 317:210-7), but these assays are not truly homogenous; i.e. they require the post-reaction addition of developing protease reagents. In addition, the applicability of this method is limited to peptides where kinase and protease specificity overlap.

[00011] There are also a few methods that are dependent on interaction of reaction products with specific multiwell plate chemistries, but these are not truly homogenous in that they require post reaction reagent additions and/or processing. Also the requirement for specialized instrumentation for processing and/or detection does not fit with the open architecture desired by most pharmaceutical HTS platforms.

[00012] Furthermore, microfluidics-based kinase assays that rely on electrophoretic separation of reaction products have been developed (Xue, Q., et al., *Electrophoresis*, 2001, 22:4000-7). In these assays, phosphorylated peptide products are electrophoretically separated from the non-phosphorylated acceptor substrates, thus eliminating the need for specialized detection reagents. However, in practice, the kinase assays are often run in multiwell plates and then the products are transferred to microfluidic devices for separation - a cumbersome process for an HTS format.

[00013] In summary, the non-generic nature of the current group transfer assays is resulting in significant expense and delays for drug discovery because of the need to develop assays for individual enzymes or small subgroups within a family. Also, because many of the current assays are based on modification and detection of specific tagged

acceptors, there is limited ability for testing different acceptor substrates. Often the tagged acceptor substrates used are different from the substrates that are phosphorylated *in vivo*, thus the physiological relevance of the assay is questionable. In addition, a major concern in the pharmaceutical industry is that because of the non-generic nature of the current assays, investigators are sometimes forced to use different methods for different kinases. However, studies have shown that there are significant differences in the pharmaceutical targets identified using different assays methods (Sills, M. A., et al., J Biomol Screen, 2002, 7:191-214), which is a significant problem for profiling inhibitor selectivity across several kinases. These shortcomings of the existing HTS assay methods for group transfer reactions are hampering the rapid analysis of important enzyme families in pharmaceutical drug discovery programs.

[00014] Other existing approaches for assaying group transfer reactions have been to enable screening of diverse chemicals as substrates for group transfer reactions by detecting the donor molecule product, because it is the same regardless of the acceptor being modified. Detection of the donor product has been thought to provide the basis of a generic assay method, ADP is always a product of a kinase reaction and phosphoadenosine-phosphate (PAP) is always the product of a sulfotransferase reaction. Detection of these products, however, is complicated because the cleaved mono- and dinucleotides cannot be differentiated from the activated donor molecules based on absorbance or fluorescence properties because for example ADP has the same fluorescence and absorbance properties as ATP. Separation of the donor product from the donor can be effected using chromatographic methods, such as thin layer chromatography or high pressure liquid chromatography, but incorporation of these methods into an HTS format is cumbersome.

[00015] To circumvent this difficulty, detection of the donor product has been achieved by using additional enzymes to generate a detectable product from the primary reaction product – the cleaved mono- or di- nucleotide; this is known as an enzyme coupled reaction. For instance, enzymes and other small molecules can be used for ADP-dependent generation of NADPH, which is detected by absorbance or fluorescence at 340nm (Walters, W. P. and Namchuk, M., Nat Rev Drug Discov, 2003, 2:259-66). An enzyme coupled reaction has also been developed for UGTs, another type of group transfer enzyme (Mulder, G. J. and van Doorn, A. B., Biochem J, 1975, 151:131-40). However, the optical interference of drug compounds with absorbance assays, especially in the ultra violet, is a widely recognized problem with this approach. Another shortcoming of this

approach is that all of the enzymes used to couple the detection are subject to potential inhibition from the chemicals being screened.

[00016] Another generic approach is to monitor ATP consumption using Luciferase as a reporter to detect protein kinase activity. An example of this method was disclosed by Crouch et al., in U.S. Pat. No. 6,599,711. Their method entailed determining the activity of a protein kinase to be tested by adding a substrate capable of being phosphorylated by the protein kinase to a solution having ATP and a protein kinase to be tested, and another solution having ATP in the absence of the kinase to be tested. The concentration or the rate of time change of ATP and/or ADP was then measured using bioluminescence. However this assay is not optimal because it relies on small decreases in a high initial signal. The need to keep ATP concentrations low to minimize background results in nonlinear reaction kinetics if assay conditions are not carefully controlled. In a related method, competition binding assays using fluorescent ATP analogs have also been developed, but these do not give a measure of enzyme catalytic activity, thus are of limited utility.

[00017] The use of enzymes that catalyze group transfer reactions into HTS assays has been hampered by the lack of universal, homogenous assay methods that are not subject to interference from molecules in pharmaceutical drug libraries. Accordingly, it would generally be desirable to provide universal methods for assaying enzymes involved in group transfer reactions that are well suited for HTS drug discovery.

BRIEF SUMMARY OF THE INVENTION

[00018] To enable facile incorporation of important group transfer enzymes into HTS assays, applicants have developed assays based on homogenous immunodetection of the donor product. The general equation for the group transfer reaction is: donor-X + acceptor \rightarrow donor-product + acceptor -X, wherein the donor-product is detected by the general detection reaction: first complex + donor-product \rightarrow second complex + displaced detectable tag. Because the donor product is the same for all enzymes that catalyze a given type of group transfer reaction, the same detection reagents can be used for all the members within a family of group transfer enzymes and with any acceptor substrate. The assay products can be detected using homogenous fluorescence or chemiluminescence methods which are not subject to significant interference or background signal from molecules in pharmaceutical drug libraries.

[00019] Thus, the present invention is summarized as methods and components thereof for detecting the activity of and screening acceptor substrates, inhibitors, or activators of enzymes catalyzing group transfer reactions to facilitate the development of

more selective and therapeutic drugs. This is accomplished through a highly selective antibody used to bind the donor product of the group transfer reaction. Antibody-antigen interactions can be detected in a number of ways that have already been described by others. The detection mode applicants have used to put the method into practice is a competitive fluorescence polarization immunoassay (FPIA), because it is well suited for pharmaceutical HTS assays. With this detection mode, enzymatically generated donor product displaces a fluorescent derivative of the donor product, called a tracer, from an antibody resulting in a decrease in tracer fluorescence polarization. The key reagents for the assay are an antibody that binds with high selectivity to the donor product, and not to the uncleaved donor molecule, and a tracer – a fluorescent derivative of the donor product that retains its structure sufficiently to bind the antibody. The invention provides a novel assay for detecting and quantifying activity for enzymes that catalyze group transfer reactions using diverse substrates. The invention also provides a method of screening for substrates, inhibitors, or activators of the group transfer reactions.

[00020] In one aspect, the invention provides a method of detecting a donor-product of a group transfer reaction, the method including reacting an activated form of a donor with an acceptor in the presence of a catalytically active enzyme; forming the donor-product and an acceptor-X; contacting the donor-product with a first complex comprising a detectable tag capable of producing an observable; competitively displacing the detectable tag of the first complex by the donor product to generate a second complex and a displaced detectable tag; and detecting a change in the observable produced by the detectable tag in the first complex and the displaced detectable tag.

[00021] In another aspect, the invention provides an antibody produced against a donor product of a group transfer reaction, wherein the antibody has the ability to preferentially distinguish between a donor-product and a donor in the presence of a high donor concentration.

[00022] In yet another aspect, the invention provides a homogeneous competitive binding assay for a donor product of a group transfer reaction, the assay includes combining the donor-product with a tracer and a macromolecule to provide a mixture, the macromolecule being specific for the donor product, the tracer comprising the donor-product conjugated to a fluorophore, the tracer being able to bind to the macromolecule to produce a detectable change in fluorescence polarization; measuring the fluorescence polarization of the mixture to obtain a measured fluorescence polarization; and comparing the measured fluorescence polarization with a characterized fluorescence polarization value, the characterized fluorescence polarization value corresponding to a known donor-product concentration.

[00023] In yet another aspect, the invention provides assay kits for characterizing a donor-product from a group transfer reaction, the assay kit including a macromolecule and a tracer, each in an amount suitable for at least one homogeneous fluorescence polarization assay for donor-product, wherein the macromolecule is a an antibody or an inactivated enzyme.

[00024] Other advantages and a fuller appreciation of specific adaptations, compositional variations, and physical attributes will be gained upon an examination of the following detailed description of the various embodiments, taken in conjunction with the appended claims.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

[00025] FIG. 1 illustrates a fluorescence polarization immunoassay (FPIA) reaction for the SULT reaction product, PAP.

[00026] FIG. 2 illustrates use of FPIA to detect and quantify UDP formation, the donor product of the UGT reaction.

[00027] FIG. 3 illustrates use of FPIA to detect and quantify ADP formation, the donor product of the kinase reaction.

[00028] FIG 4 describes a strategy for iterative co-development of reagents for SULT1E1 FPIA: anti-PAP antibody and fluorescently labeled PAP detectable tag.

[00029] FIGS. 5 A-B show titration or competitive displacement curves for uridine nucleotides using a polyclonal antibody raised against UDP/UTP and a commercially available tracer molecule (Alexa-UTP).

[00030] FIG. 6 shows the synthesis of PAP antigens.

[00031] FIGS. 7 A-C show components of PAP tracer synthesis.

[00032] FIGS. 8 A-C show representative final tracer structures.

[00033] FIG. 9 shows binding isotherms for anti-PAP antibodies and PAP-fluorescein tracers.

[00034] FIG. 10 shows competitive displacement of two different tracers from Ab 3642 by PAP and PAPS.

[00035] FIGS. 11 A-B show the competition curves with two Anti-PAP antibody/Tracer combinations.

[00036] FIG. 12 shows the effect of PAPS concentration on detection of enzymatically generated PAP.

[00037] FIGS. 13 A-F show a continuous FPIA-based detection of SULT activity with diverse substrates.

[00038] FIG. 14 shows a comparison of SULT1E1 acceptor substrate profiles determined using the FPIA-based assay and the ³⁵S-PAPS radioassay.

[00039] FIG. 15 shows inhibition of SULT1E1 by 2,6 Dichloro-4-nitrophenol (DCNP) measured with the FPIA-based assay.

[00040] Before an embodiment of the invention is explained in detail, it is to be understood that the invention is not limited in its application to the details set forth in the following description. The invention is capable of other embodiments and of being practiced or being carried out in a variety of ways. Also, it is to be understood that the phraseology and terminology used herein is for the purpose of description and should not be regarded as limiting in any way.

DETAILED DESCRIPTION OF THE INVENTION

[00041] The present invention broadly relates to novel assay methods for detecting and quantifying the donor-product or the catalytic activities generating the donor-product from group transfer enzymes using diverse substrates. The invention also provides the antibodies specific to the donor product and assay kits for practicing the invention in a high-throughput screening format. The general equation for the group transfer reaction includes a donor-X + acceptor → donor-product + acceptor -X, wherein the donor-product is detected by the general detection reaction: first complex + donor-product → second complex + displaced detectable tag. Thus, in accordance with the invention, a highly selective antibody is used to bind the donor product of the group transfer reaction, and this binding event is detected, using an immunoassay, such as for example a competitive binding FPIA which is well suited for pharmaceutical HTS assays. By using this detection mode, enzymatically generated donor product displaces a tracer, from an antibody resulting in a decrease in tracer fluorescence polarization. The key reagents for the assay are antibody that binds with high selectivity to the donor product, and not to the uncleaved donor molecule, and a tracer – a fluorescent derivative of the donor product that retains its structure sufficiently to bind the antibody.

[00042] In particular the invention provides a universal assay method in that a single set of detection reagents can be used for all of the enzymes in a given family of group transfer enzymes and all acceptor substrates for that family. Because of its universal nature, the invention will accelerate the incorporation of SULTs, protein kinases and other group transfer enzymes into HTS screening. For example, there are eleven known SULT isoforms, using the method of the present invention, all eleven of the SULT isoforms may be screened for their

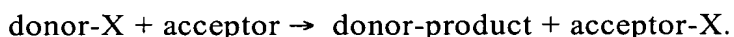
ability to sulfate diverse compounds in the same experiment using the same detection reagents, protocol and instrumentation. This is an important capability because enzymes that catalyze xenobiotic conjugation (e.g., SULTs and UGTs) have very broad acceptor substrate specificity. With respect to kinases, there is even more diversity within the enzyme family. There are over 400 protein kinases in humans, and there is great diversity in their acceptors substrate specificity such that either physiological protein acceptor substrates as well as short peptides may be used. As such, a number of protein kinases may be used in the assay of the invention using their diverse acceptor substrates and screened for inhibitors using the same detection reagents, protocol and instrumentation. For these reasons and others provided below, the FPIA based donor product detection assays of the invention for group transfer reactions such as SULT, UGT and kinases, among others are very well suited for automated HTS applications.

[00043] Accordingly, the present invention will now be described in detail with respect to such endeavors; however, those skilled in the art will appreciate that such a description of the invention is meant to be exemplary only and should not be viewed as being limiting on the full scope thereof.

DEFINITIONS

Certain terms used herein are intended to have the following general definitions:

[00044] The term “group transfer reaction” as used herein refers to the general reaction:



[00045] Representative group transfer reactions are shown as follows:

Kinase reaction: $\text{ATP} + \text{acceptor} \rightarrow \text{ADP} + \text{acceptor-PO}_4$;

UGT reaction: $\text{UDP-glucuronic acid} + \text{acceptor} \rightarrow \text{UDP} + \text{acceptor-glucuronic acid}$;

SULT reaction: $\text{acceptor-XH} + 3'\text{-phosphoadenosine } 5'\text{-phosphosulfate} \rightarrow \text{acceptor-SO}_4 + 3'\text{-phosphoadenosine } 5'\text{-phosphate} + \text{H}^+$;

Methyltransferase reaction: $\text{s-adenosylmethionine} + \text{acceptor} \rightarrow \text{acceptor-CH}_3 + \text{s-adenosylhomocysteine}$; and

Acetyl transferase reaction: $\text{acetyl CoenzymeA} + \text{acceptor} \rightarrow \text{acceptor-COCH}_3 + \text{CoenzymeA}$.

[00046] Group transfer reactions in-part such as sulfation by SULTs, phosphorylation by kinases and UDP-glucuronidation by UGTs are suitably applicable to the methods of the invention because one can isolate antibody/detectable tag pairs for the donor products, which are PAP, ADP and UDP, respectively. Also group transfer reactions are generally involved in a

number of biological processes, such as hormone biosynthesis and function; enzyme regulation and function; and xenobiotic metabolism.

[00047] The term “universal assay” and “generic assay” are used interchangeably to refer to a method whereby all members of the group transfer reaction enzyme family and all of their acceptor substrates can be detected with the same assay reagents.

[00048] The term “covalent adduct” refers to the moiety that is transferred from the donor molecule to the acceptor in a group transfer reaction; sulfonate, phosphate, and glucuronic acid respectively for SULTs, kinases, and UGTs.

[00049] The term “donor-product” as used herein refers to the product of a group transfer reaction that is the fragment of the donor molecule that is generated when the covalent adduct is transferred to acceptor. Often it is a nucleotide (naturally occurring or synthetic) such as a PAP, UDP or ADP; or a non-nucleotide such as a s-adenosylhomocysteine, nicotinamide or a CoenzymeA. The donor-product is detected by a general reaction including a first complex + donor-product → second complex + displaced detectable tag.

[00050] The term “tracer” as used herein refers to a displaced detectable derivative or tag of a donor product that retains its structure sufficiently to bind to a specific antibody.

[00051] The term “donor” as used herein refers to a substrate for an enzyme catalyzing a group transfer reaction that carries the activated covalent adduct. Examples of suitable donors include not only nucleotides, but also s-adenosyl methionine and acetyl-CoA, among others.

[00052] The term “donor-X” is another term for donor molecule in which X represents the covalent adduct.

[00053] The term “acceptor” as used herein refers to a substrate for an enzyme catalyzing a group transfer reaction to which the covalent adduct becomes covalently attached, wherein the substrate is a polypeptide, a protein, a nucleic acid, a carbohydrate, a lipid or a small molecule substrate such as a steroid or an amino acid.

[00054] The term “acceptor-X” as used herein refers to a reaction product in which X is the covalently bound covalent adduct; wherein the covalent adduct includes at least one of a phosphate, a sulfate, a carbohydrate, a naturally occurring amino acid, a synthetically derived amino acid, a methyl, an acetyl, or a glutathione moiety, and a combination thereof. The covalent adduct is capable of altering either the function, the stability, or both the function and the stability of the acceptor substrate.

[00055] The term “catalytically active enzyme” as used herein refers to at least one of a sulfotransferase, a kinase, a UDP-glucuronosyltransferase, a methyl transferase, an acetyl transferase, a glutathione transferase, or a ADP-ribosyltransferase.

[00056] The term “catalytic activity” as used herein refers to a chemical catalytic activity, an enzymatic activity, or a combination thereof.

[00057] The term “first complex ” as used herein refers to a complex having a macromolecule (i.e., an antibody or an inactivated enzyme) and a detectable tag.

[00058] The term “second complex ” as used herein refers to a macromolecule and the donor product wherein the detectable tag is competitively displaced by the donor-product.

[00059] The term “observable” as used herein refers to detectable change in fluorescence, fluorescence intensity, fluorescence lifetime, fluorescence polarization, fluorescence resonance energy transfer (FRET) or chemiluminescence of the second complex or the displaced detectable tag and a combination thereof to obtain a measured observable. The measured observable is compared with a characterized observable, wherein the characterized observable corresponds to the first complex.

[00060] The term “detectable tag ” as used herein refers to a fluorescent or chemiluminescent tracer, which is conjugated to a donor product. Fluorescence is the preferred mode of detection for the invention. A suitable detectable tag may be produced by conjugating for example, a chemiluminescent tag or a fluorophore tag, to the donor product molecule in such a way that it does not interfere significantly with antibody binding (i.e., most likely attached via the adenine portion of the nucleotide). Fluorophores applicable to the methods of the present invention include but are not limited to fluorescein, rhodamine, BODIPY, Texas Red, and derivative thereof known in the art. Rhodamine conjugates and other red conjugates may be synthesized and optimized as detectable tags because their higher wavelength emission is less subject to interference from autofluorescence than the green of fluorescein.

[00061] Chemiluminescent tags applicable to the invention include Lumigen TMA-6 and Lumigen PS-3 (Lumigen, Inc., Southfield, MI) which have adequate chemiluminescence quantum yield. These reagents possess an easily measured signal by virtue of an efficient chemiluminescent reaction with a predictable time course of light emission. Furthermore, chemiluminescent tags contribute little or no native background chemiluminescence. Also, measurement of light intensity is relatively simple, requiring only a photomultiplier or photodiode and the associated electronics to convert and record signals. In addition, chemiluminescent signals can be generated in an immunoassay using enzyme fragment complementation methods, where the detectable tag would be the donor product conjugated to fragment A of a reporter enzyme, and its displacement from antibody by the donor product generated in the group transfer reaction would allow it to associate with fragment B of the enzyme resulting in formation of a catalytically active reporter enzyme capable of acting on a

chemiluminescent substrate. This method has been described using β -galactosidase as a reporter enzyme in U.S. patent no. 4,708,929.

[00062] The term “immunoassay” as used herein may refer to a number of assay methods wherein the product is detected by an antibody such as for example a homogenous assay, homogeneous fluorescence immunoassay, a homogeneous fluorescence intensity immunoassay, a homogeneous fluorescence lifetime immunoassay, a homogeneous fluorescence polarization immunoassay (FPIA), a homogeneous fluorescence resonance energy transfer (FRET) immunoassay or a homogenous chemiluminescent immunoassay, or a non-homogenous assay such as enzyme-linked immunoassay (ELISA) and a combination thereof.

[00063] The term “fluorescence polarization immunoassay” or “FPIA” as used herein refers to an immunoassay for detecting the products of group transfer reactions. Fluorescence polarization (FP) is used to study molecular interactions by monitoring changes in the apparent size of fluorescently-labeled or inherently fluorescent molecules (Checovich, W. J., et al., Nature, 1995, 375:254-6; Owicki, J. C., J Biomol Screen, 2000, 5:297-306). When a small fluorescent molecule (tracer) is excited with plane polarized light, the emitted light is largely depolarized because the molecule rotates rapidly in solution during the fluorescence event (the time between excitation and emission). However, if the tracer is bound to a much larger receptor, thereby increasing its effective molecular volume, its rotation is slowed sufficiently to emit light in the same plane in which it was excited. The bound and free states of the fluorescent molecule each have an intrinsic polarization value, a high value for the bound state and a low value for the free state. In a population of molecules, the measured polarization is a weighted average of the two values, thus providing a direct measure of the fraction of the tracer molecule that is bound. Polarization values are expressed as millipolarization units (mP), with 500mP being the maximum theoretical value.

[00064] In practice, small molecules like fluorescein have polarization values of approximately 20 mP, and when bound by an antibody their polarization increases by 100 to 400mP. In a competitive FPIA, a fluorescent tracer may be displaced from binding to antibody by the donor product, as shown in Figure 1. The signal is proportional to the difference in the bound versus free tracer fractions, thus both the dynamic range and the sensitivity of the assay are dependent upon the affinity of the antibody for the tracer and the donor product. To establish a suitable dynamic range for an FPIA, approximately 70-80% of the tracer must be bound to antibody in the absence of competitor.

[00065] For example, figure 1 shows a schematic of a competitive FPIA for the SULT reaction product PAP in which the PAP produced from the SULT reaction competes with the tracer (fluorescently tagged PAP), for binding to anti-PAP antibody. In this format, the starting polarization of the tracer is high because it is almost all bound to antibody, and it decreases as the reaction proceeds and the tracer is displaced from the antibody. The amount of PAP produced in a SULT reaction can be quantified by using the following equation:

$$\log [\text{PAP product}] = \log \frac{(\text{highest polarization} - mP_{\text{observed}})}{(mP_{\text{observed}} - \text{lowest polarization})} + \log IC_{50}.$$

[00066] In the enzymatic assay encompassed by the present invention, the PAP for SULTs or ADP for kinases is produced in stoichiometric amounts with the sulfated product or phosphorylated peptide, respectively. Thus the use of a standard curve for PAP or ADP among other donor products will allow a direct measure of enzyme turnover.

[00067] The term “high throughput screening” or “HTS” as used herein refers to the testing of many thousands of molecules (or test compounds) for their effects on the function of a protein. In the case of group transfer reaction enzymes many molecules may be tested for effects on their catalytic activity. HTS methods are known in the art and they are generally performed in multiwell plates with automated liquid handling and detection equipment; however it is envisioned that the methods of the invention may be practiced on a microarray or in a microfluidic system.

[00068] The term “library” or “drug library” as used herein refers to a plurality of chemical molecules (test compound), a plurality of nucleic acids, a plurality of peptides, or a plurality of proteins, and a combination thereof. Wherein the screening is performed by a high-throughput screening technique, wherein the technique utilizes a multiwell plate or a microfluidic system.

[00069] The terms “binding molecule” or “macromolecule” as used herein refers to an antibody or an inactivated enzyme.

[00070] The term “antibody” as used herein refers to a monoclonal, a polyclonal or recombinant antibody. The antibody is produced against a donor-product of a group transfer reaction and is able to preferentially distinguish between a donor-product and a donor in the presence of a high donor concentration. The antibody also exhibits specificity towards at least one of a phosphate portion of a nucleotide, (i.e., an ability to distinguish between a 5'-phosphate, a 5'-phosphosulfate, a 5'-diphosphate and a 5' –triphosphate). For example, in the SULT reaction, the antibody of the present invention differentiates with high stringency between

PAP and PAPS. The donor-product molecule for the SULT reaction, PAPS, differs only by the addition of a sulfate group linked to the 5' terminal phosphate. This may seem like a relatively small structural difference, but the demonstrated ability of antibodies to discriminate between molecules that differ by a single phosphate – which is very similar in size and structure to a sulfate group – provides an important feature of the present invention. For example, an antibody may be raised against the ribosyl phosphate portion of the molecule. Furthermore, the FPIA-based SULT assay method of the present invention suitably requires an antibody that specifically binds the reaction product PAP in the presence of excess PAPS.

[00071] In practicing the invention with respect to kinases, antibodies that specifically recognize ADP and not ATP may be generated in animals or by *in vitro* recombinant methods using ADP conjugated to a carrier protein in such a way that the phospho-ribosyl portion of the molecule is exposed, but the adenine portion is largely hidden from immunoreactivity. In generating antibodies, it may be helpful to use a nonhydrolyzable analog of ADP, such as one containing a methylene or sulfur group bridging the alpha and beta phosphates in order to prevent hydrolysis of the immobilized hapten by nucleotidases or phosphatases in the immunized animals. Alternatively, with respect to methyltransferases and acetyltransferases, antibodies that specifically recognize small molecules other than nucleotides, respectively S-adenosyl homocysteine and Coenzyme A are used.

[00072] The term “inactivated enzyme” as used herein refers to a binding molecule that may bind to the donor product. In the case of a kinase, the ADP is the donor product and an inactivated nucleoside diphosphate (NDP) kinase may be the binding molecule. It is known that in many enzymes, separate domains are involved in binding and in catalysis. A selective destruction of the catalytic activity with preservation of the binding properties by genetic engineering, allosteric inhibition, or other chemical means such as taking out cofactors, heme groups, etc. may enable enzymes, particularly multi-subunit enzymes to function as specific carriers for their substrates that are no longer able to chemically modify these substrates.

[00073] The term “antibody-detectable tag pair” as used herein refers to an anti-donor product antibody and detectable tag (fluorescent-donor product) molecule that allow detection of donor product produced in a group transfer reaction. A suitable dissociation constant for binding of the antibody/detectable tag pair is $1 \times 10^{-7} \text{M}$ or lower, resulting in a minimal fluorescence polarization shift of 100mP relative to the unbound detectable tag, and with minimal cross reactivity to the donor molecule at reaction concentrations. The optimal antibody-detectable tag pair may be identified by testing a number of different antibodies generated using different sites of attachment to the donor molecule, different linkers to the

carrier protein, and including some non-hydrolyzable analogs for interaction with a set of detectable tags generated by varying the chemistry of donor molecule attachment to a fluorophore. The changes in fluorescence or chemiluminescence of the detectable tag upon interaction with an antibody that could have a fluorophore for example attached to it; may be used as a measure of their interaction.

[00074] The term “linker” as used herein refers to spacer arm structures. There are short linkers (i.e., carbamoyl and aminoethyl groups) that will sterically minimize the accessibility of the adenine ring and longer six carbon linkers that will allow more flexible presentation of the antigen. The linker molecule affects detectable tag characteristics in a number of important ways that impact both its antigenic and fluorescence properties. There is generally a balance that must be struck between separating the antigen from the fluorophore enough to allow unhindered interaction with antibody without creating too much freedom of motion for the fluorophore. The former result in lowered affinity antibody binding and in quenching of the fluorophore, whereas the latter reduces the polarization shift upon antibody binding, thereby reducing the dynamic range of the assay. Different linkers can be interchanged using relatively simple chemistry, thus the linker is varied in a number of ways in efforts to optimize the antigenic and fluorescence properties of the detectable tag. Furthermore, approaches may be employed similar to those described for PAP conjugation of an antibody involving heterobifunctional linkers to first introduce spacer arms and/or aromatic substituents onto PAP, followed by reaction with reactive fluorescein derivatives. This approach greatly expands the range of possible linker structures.

METHODS AND MATERIALS

[00075] The following experimental protocols of the invention are not limited to the particular methodology, protocols, antibodies, enzymes, detectable tags, among other reagents described, as these may vary depending on the group transfer reaction. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention.

Development of Assay Reagents

[00076] In developing a successful HTS assay format for the invention the affinity and specificity of assay reagent interaction, and the resultant changes in detectable tag fluorescence properties ultimately define the overall performance of the assay, including sensitivity, dynamic range, and signal to noise ratios. As such, to optimize this interaction, applicants have provided

an iterative co-development approach as shown in Figure 4 to most efficiently achieve detection, quantitation and screening of group transfer reaction products.

[00077] In particular, figure 4 describes a strategy for iterative co-development of reagents for a SULT donor product FPIA: anti-PAP antibody and fluorescently labeled PAP detectable tag. Figure 4 provides that to produce diverse antigens for immunization of rabbits, PAP may be conjugated to carrier protein (BSA or KLH) via attachment through the C2, C8 and N6 amino group of adenine using linker molecules of varying lengths as described below. The same PAP-linker-NH₂ intermediates used for antibody conjugation may be used to synthesize fluorescein-PAP conjugates to test as detectable tags. The resulting matrix of antibodies and detectable tags may be tested for binding using FP assays, and the pairs that exhibit high affinity binding and maximal FP shifts may be used as the basis for further optimization of tracer characteristics. Optimized Antibody-detectable tag pairs may be tested for detection of PAP production in sulfation reactions with SULT1E1 or another SULT isoform.

[00078] Also, it is envisioned that several PAP antigens and detectable tags may be synthesized by conjugating the nucleotide to carrier protein and fluorescein, while retaining an overall structural bias that maximizes antibody recognition of ribosyl-phosphate moieties of the PAP molecule and minimize crossreactivity with PAPS. Antibodies generated from the PAP antigens may be tested for interaction with the detectable tags to identify the combinations that exhibit optimal binding and fluorescence properties of the novel assay encompassed by the present invention.

Generation of High Affinity Antibodies

[00079] In accordance with methods of the present invention, one skilled in the art may readily prepare a variety of specific antibodies (i.e., polyclonal, monoclonal or recombinant) against antigenic nucleotides such as the ribosyl-phosphate portion of PAP or other chemical molecules such as Coenzyme A.

[00080] In general polyclonal antibodies against a range of PAP antigens are suitably generated in rabbits. The injection of animals and collection of serum may be performed according to the following protocol. Three rabbits may be immunized with each of the antigens that may be developed. The yields of antiserum from a single rabbit (~100ml) are suitable for many thousand to millions of FPIA assays depending on the titer, affinity, and the multivalent nature of polyclonal-antigen interactions resulting in very high affinity binding. As such, for

these reasons polyclonals are frequently used for FPIAs (Nasir, M. S. and Jolley, M. E., Comb Chem High Throughput Screen, 1999, 2:177-90).

[00081] Also, encompassed within the scope of the present invention are monoclonal antibodies which may be produced according to the methods provided in previously published protocols (Harlow, E. L., D., 1999), which are fully incorporated herein by reference. Also, recombinant single chain antibodies, may be generated using the *in vitro* combinatorial evolution and display methods previously published (Schaffitzel, C., et al., J Immunol Methods, 1999, 231:119-35; Breitling, F., Dubel, S., 1999). Examples of the preparation of recombinant antibodies are described in U.S. Patent Nos.: 5,693,780; 5,658,570; 5,876,961 (fully incorporated by reference herein).

[00082] In order to help elicit an immune response, bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH) may be used as carrier proteins for conjugation of the PAP antigen. KLH antigens generally elicit a stronger immune response in mammals, but also tend to be less soluble than BSA conjugates (Harlow, E. L., D., 1999). PAP may be attached to both carrier proteins via the C2, C8 and N6 amino group of adenine as shown in Figures 7-8. In accordance with the scope of the present invention, development of the FPIA-based SULT assay method requires an antibody that specifically binds the reaction product PAP in the presence of excess PAPS. More suitably, the antibody is capable of recognizing the ribosyl phosphate portion of the PAP molecule, and to differentiate between the 5'-phosphate of PAP and the 5'-phosphosulfate of PAPS.

Conjugation Methods

[00083] It is known in the art that conjugation to adenine will minimize antibody cross reactivity with PAPS that could occur via binding to the adenine moiety (Goujon, L., et al., J Immunol Methods, 1998, 218:19-30; Horton, J. K., et al., J Immunol Methods, 1992, 155:31-40; Bredehorst, R., et al., Biochim Biophys Acta, 1981, 652:16-28), and the site of hapten conjugation also can affect the affinity of the resulting antibodies (Crabbe, P., et al., J Agric Food Chem, 2000, 48:3633-8). The hapten conjugation chemistries used are well described single or two step synthetic approaches that have been used extensively for conjugating adenine nucleotides (Brodellius, P., et al., Eur J Biochem, 1974, 47:81-9; Lindberg, M. and Mosbach, K., Eur J Biochem, 1975, 53:481-6; Camaioni, E., et al., J Med Chem, 1998, 41:183-90). All of the starting materials are commercially available, and it is understood that the reactions may proceed in good yield. These conjugation methods are described below in further detail. In accordance

with the methods of the present invention, it is envisioned that the present invention may encompass but may not be limited by the following hapten conjugation chemistries.

Attachment to N6 amino group

[00084] The carbamoyl linkage: N6-carboxymethyl-PAP may be generated by alkylation of PAP with iodoacetic acid to form the 1-carboxymethyl analog, which rearranges in base to form the N6-carboxymethyl analog (Lindberg, M. and Mosbach, K., Eur J Biochem, 1975, 53:481-6). The N6-carboxymethyl-PAP may be conjugated to carrier protein using carbodiimide coupling enhanced with N-hydroxysulfosuccinimide. A similar approach could be used to generate high specificity 5'-AMP antibodies as described in previously published protocols (Bredehorst, R., et al., Biochim Biophys Acta, 1981, 652:16-28).

[00085] The C6 linkage: N6-aminohexyl-PAP (Sigma/Aldrich, St. Louis, MO) may be directly conjugated to carrier proteins by a) converting protein amino groups (mostly lysines) to reactive thiols using N-succinimidyl S-acetylthioacetate (Pierce, Rockford, IL) and b) conjugating the terminal amine group on the PAP linker to the protein thiols using heterobifunctional amine-thiol reactive linkers (Pierce, Rockford, IL). It is noteworthy that the N6 amino group of adenosine is for practical purposes unreactive; for instance it reacts very poorly with succinimidyl esters.

Attachment to adenosine C2

[00086] The 2-chloroadenosine may be phosphorylated by reaction with phosphorous oxychloride followed by phosphorous trichloride to produce 3', 5'-bisphospho-chloroadenosine. Subsequent reaction with ethylenediamine and diaminoethane and purification by anion exchange chromatography may be used to produce the 2-aminoethyl- and 6-aminohexyl-analogs of PAP at the 2 position of adenine (Brodellus, P., et al., Eur J Biochem, 1974, 47:81-9). It is envisioned that these molecules may react with carrier proteins using heterobifunctional linkers as described above.

Attachment to adenosine C8

[00087] The C2 and C6 linkages to the 8 position of PAP may be generated using the same strategy described for the C2 position, except the starting material may be 8-bromoadenosine (Sigma/Aldrich). Also, 8-azido-PAP (ICN, Costa Mesa, CA) may be directly bound to carrier proteins by UV irradiation.

[00088] Thin layer chromatography (TLC) may be used to monitor changes in the adenine ring absorption spectrum. Anion exchange (Dowex resin) and phase separations may be used for antigen purification. To assure high antigen purity, the final PAP-linker molecules to be used for conjugation to carrier proteins may be purified by HPLC and their identity verified by mass spectra and NMR analysis. The N6-carboxymethyl-PAP may be conjugated to methylated BSA, since use of the methylated carrier protein has been reported to prevent carbodiimide reaction with the ribose ring (Bredehorst, R., et al., *Biochim Biophys Acta*, 1981, 652:16-28).

[00089] The other PAP analogs all tend to have a reactive amine at the end of the linker, which may be used for conjugation to BSA and KLH using more specific amine and thiol chemistry, thereby avoiding reaction with the ribose. The conversion of protein amines to thiols and linking via heterobifunctional linkers may result in a higher density of antigen conjugation than direct conjugation to cysteines because there are many more reactive lysines than cysteines in the carrier proteins. PAP density on the carrier protein may be determined by spectrophotometric analysis for the adenine ring and conjugation chemistries may be adjusted if needed to obtain a high density (>10 PAP per protein molecule) and thus insure a robust immune response. It is envisioned that about 8-10 different antigens may be synthesized in all, some with very short linkers such as the carbamoyl and aminoethyl groups that will sterically minimize the accessibility of the adenine ring and also with longer six carbon linkers that will allow more flexible presentation of the antigen.

Synthesis and purification of fluorescent PAP molecules

[00090] In general, an FPIA detectable tag molecule can be divided into three different structural components: the antigen, the fluorophore, and the linker used to join them; an additional key structural variable is the site of attachment of the linker to the antigen. It is understood by one skilled in the art that suitable antigens, fluorophores and linkers are not limited to what is specifically described herein. However, in a suitable aspect of this invention the fluorophore, fluorescein, may be used to conjugate it to the same sites on adenosine used for antigen conjugation to carrier protein. The same PAP-linker molecules that are used for conjugation to carrier proteins may be used for conjugation to several amine-reactive fluorescein derivatives. The use of a range of reactive fluoresceins may introduce additional structural diversity in the linker region. For instance, fluorescein succinimidyl esters are available with different length spacer arms, and DTAF (4,6-Dichlorotriazinylamino fluorescein) contains a bulky planar substituent adjacent to the site of attachment. Likewise, the introduction of an aromatic ring to AMP near the site of carrier protein conjugation (N6-benzyl AMP) enhances its

affinity for antibody by 40-fold relative to unmodified AMP (Bredehorst, R., et al., *Biochim Biophys Acta*, 1981, 652:16-28). Briefly, various PAP-linker molecules with amino termini may be reacted with amine-reactive rhodamine, Texas Red, and other red fluorophors and the resulting PAP- fluorophors conjugates may be tested for antibody binding affinity and changes in fluorescence polarization.

[00091] Purification of PAP-conjugates can be performed by thin layer chromatography (TLC). TLC is used to separate reaction products because it allows several separations to be run in parallel, permits identification of fluorescent products by visualization and, provides sufficient quantities of fluoresceinated conjugates for thousands of binding assays in a single chromatography run. Thus for pilot studies on fluorescent detectable tag molecules, TLC is superior to alternative separation methods like HPLC. Upon completion of the chromatography and elution of the PAP-fluorophore conjugates, the conjugates may be analyzed for intensity, basal polarization (absence of antibody), stability, binding to anti-PAP antibodies, and competition by unlabeled PAP and PAPS, as described below.

Parameters affecting the antibody-detectable tag interaction

[00092] In order to create the most sensitive assay method for detecting, quantifying, and screening products of group transfer methods, the parameters affecting the affinity of the antibody-detectable tag interactions is carefully monitored. This is important, for example, in the case of SULT1E1 where the potent substrate inhibition exhibited by some acceptor substrates, may limit the amount of product that can be generated. The affinity required for the novel SULT assay encompassed by the present invention is estimated by considering the limiting case of the high affinity, inhibitory substrate, estradiol. In general, the K_i for estradiol is 80nM and the K_m is about 20nM (Zhang, H., et al., *J Biol Chem*, 1998, 273:10888-92), so using 50nM estradiol in the assay and adjusting enzyme and reaction time so that 10% of the substrate is consumed – resulting in formation of 5nM PAP - is a good compromise that may allow measurement of initial velocity without causing significant inhibition.

[00093] To allow detection of 5nM PAP the required affinity for antibody binding to fluorescent-PAP (and unconjugated PAP) may be approximately 25nM. Most antibody-antigen interactions with either polyclonal or monoclonal antibodies have a K_d between 1nM and 1 μ M (Harlow, E. L., D., 1999). For instance, the anti-phosphotyrosine antibodies used in FPIA-based kinase assays, exhibit subnanomolar affinity for phosphorylated peptides and their fluorescent conjugates (Parker, G. J., et al., *J Biomol Screen*, 2000, 5:77-88), and the anti-AMP polyclonal antibody described above bound to the nucleotide with an affinity of 100nM (Bredehorst, R., et

al., *Biochim Biophys Acta*, 1981, 652:16-28). It is difficult to predict how the structure of the antigen will affect the affinity of the resulting antibodies, but the carrier protein and mode of conjugation both can have a profound effect (Crabbe, P., et al., *J Agric Food Chem*, 2000, 48:3633-8; Signorini, N., et al., *Chem Res Toxicol*, 1998, 11:1169-75; Oda, M. and Azuma, T., *Mol Immunol*, 2000, 37:1111-22). Accordingly, antibodies may be generated using PAP conjugated to two different proteins via different sites on PAP and using different length linker regions.

[00094] Another factor affecting the sensitivity of PAP detection is poor substrates that are sulfated very slowly even at high concentrations. In these cases, the amount of SULT1E1 enzyme required becomes a limiting factor because of cost considerations. Using a substrate that is turned over at 1% the rate of the V_{max} with estradiol, it may require 30ng of SULT1E1 to generate 5nM PAP – a suitable detection limit in about 30 minutes. This is well within the acceptable range, as it may mean that a 100mg batch of enzyme may support more than three million reactions.

[00095] Furthermore, the difference in polarization of the probe in the free and bound states defines the total “spread” or dynamic range for the assay. A change of less than 50 mP may be sufficient for semi-quantitative detection of SULT1E1 activity, but a change of 100 mP or greater may provide both much greater flexibility in designing the assay format, and more quantitative kinetic information. Accordingly, polarization is proportional to molecular volume, and the change in effective volume upon binding of an antibody (150 kDa) to a small molecular weight fluorophore may be expected to cause an increase of at least 300 mP. However, there are other factors that can affect the observed polarization of both the free and bound detectable tag, such as, the fluorophore is attached to PAP by a flexible spacer arm. Thus, their maximum polarization is a function of the extent to which antibody binding decreases the mobility of the spacer and fluorophore; detectable tag “propeller effects” can lead to relatively low polarization values even for protein-bound fluorophores.

[00096] An additional consideration is whether there may be changes in the intensity of the fluorophores upon binding to receptor, including quenching from amino acid functional groups with overlapping spectra. Polarization is independent of fluorescence intensity (Owicki, J. C., *J Biomol Screen*, 2000, 5:297-306); however, because it is a ratio of intensity measurements taken in two planes, significant quenching may affect the antibody detectable tag interaction and result in decreased assay sensitivity. The changes in the fluorescence properties of a detectable tag when it becomes bound by antibody are a function of both interacting molecules and are difficult to predict. Thus, it is envisioned that different antibodies and

detectable tag molecules will be tested in a matrix fashion. Further optimization of the detectable tag structure or the assay buffer to limit mobility of the bound detectable tag or to reduce quenching is also envisioned.

Identification of the optimal antibody/detectable tag pair(s)

[00097] In order to identify optimal antibody/detectable tag pairs, a diverse set of fluorescein derivatives and linkage chemistries are generally evaluated. The synthesis and purification methods described above result in the isolation of several fluorescent products per reaction, so that at least a hundred fluorescent conjugates may be evaluated, requiring thousands of individual FP assays. The homogenous nature of FP assays and the availability of multiwell instruments makes the screening efforts very rapid; the rate-limiting steps are usually synthesis and purification, not testing for Antibody-detectable tag binding properties. The antibody/detectable tag testing may be carried out in a Tecan Ultra multiwell FP reader in Tris or phosphate buffer in the presence of a low concentration of carrier protein (0.01% Bovine Gamma Globulin) to prevent non-specific interactions. The initial screen involves looking for the following desirable properties: low polarization in the absence of antibody ($<100\text{mP}$); high affinity binding to the anti-PAP antibodies ($K_d < 100\text{nM}$); maximal difference in polarization between the bound and free states ($\Delta \text{mP} > 100\text{mP}$); minimal fluorescence quenching upon binding antibody; selectivity for PAP (i.e., competitive displacement by PAP and not by PAPS or other adenine nucleotides); rapid association/dissociation kinetics; and lack of interaction with SULT1E1 or other assay components.

[00098] For initial antibody binding assays, each of the purified detectable tags may be quantified by measuring fluorescence intensity with the assumption that the intensity of the conjugated fluorescein is not quenched. This is not always the case, but those with significant quenching may be eliminated through the binding analysis because they appear to have low binding affinity. Antibody/detectable tag pairs with promising affinity ($K_d < 100\text{nM}$) and fluorescence properties may be tested for the specificity of binding by competition with unlabeled PAP and PAPS and other nucleotides. Those Antibody/detectable tag pairs with the most desirable FPIA properties may be examined in more detail to assess whether any changes in extrinsic conditions such as the addition of salt or detergent may improve their sensitivity or dynamic range. Additional detectable tags may be optionally synthesized and tested based on the structure-activity relationships observed and the predictions of computer-generated structural models.

APPLICABILITY OF THE INVENTION

[00099] The methods of the invention, as described in the examples below can be used as a measure of enzymatic activity in group transfer reactions. The methods are of particular importance in the pharmaceutical industry since they enable the analysis of group transfer enzymes in high throughput screening laboratories, e.g. for identification of drugs that can act as enzymatic modulators, especially inhibitors, and for determining how potential drug molecules are metabolized.

[000100] There is an increasing number of group transfer enzymes that are being targeted for the development of new therapeutics. Kinases are currently of highest interest, but SULTs, UGTs, methyltransferases, acetyltransferases, and ADP-ribosyltransferases are also being targeted. As this list increases it will become impractical and extremely expensive to use specific tests for each enzyme, or for small subgroups of enzymes that modify the same acceptor substrate. The assays and kits of the present invention allow for the detection of all of the enzymes in a given group transfer enzyme family and provide a common end point detection system for all of them. This allows for greater ease of use, particularly in high throughput screening laboratories, where robots can be set up with all the detection reagents and the various group transfer enzymes and potential substrates or inhibitors of interest can be tested in a matrix fashion.

[000101] In addition, the involvement of group transfer reactions in the conjugative metabolism of drugs and other xenobiotics is an important determinant in the level of their pharmacodynamics, pharmacokinetics and side effects. In this regard, the assays and kits of the present invention allow for the screening of potential drug molecules with SULTs, UGTs, methyltransferases and acetyltransferases in an HTS format. There are many different xenobiotic conjugating isoforms, and because of the generic nature of the method, the kits of the present invention would greatly simplify their testing in a matrix fashion.

[000102] Thus, the general methods of the invention may be applied to a variety of different group transfer-related enzymatic processes, such as steroid hormone biosynthesis and function, xenobiotic metabolism, enzyme receptor regulation, and signal transduction in an effort to contribute to an integrated drug discovery approach discussed below.

Sulfation in drug discovery

[000103] Sulfation is a ubiquitous covalent modification used to regulate the levels and activities of endogenous hormones and xenobiotics. In humans it is catalyzed by a

family of eleven different sulfotransferase enzymes (SULTs), each with different, but overlapping substrate specificity and tissue distribution (Strott, C. A., *Endocr Rev*, 2002, 23:703-32). Endogenous substrates for sulfation include many important signaling molecules, such as steroid hormones, catecholamines, and thyroid hormones; xenobiotics that are sulfated include drugs, promutagens and environmental endocrine disruptors (Strott, C. A., *Endocr Rev*, 2002, 23:703-32; Glatt, H., et al., *Mutat Res*, 2001, 482:27-40; Coughtrie, M. W. and Johnston, L. E., *Drug Metab Dispos*, 2001, 29:522-8). Sulfation is reversible and can change the activity of signaling molecules, often by altering their affinity for receptor proteins, thus it serves as an on-off switch for receptor ligands, much as phosphorylation serves that role for proteins. Moreover, the role of sulfation in xenobiotic metabolism is intertwined with its involvement in the regulation of hormonal signaling and cell homeostasis. For instance, sulfation regulates the activity of endogenous ligands for specific neurotransmitter receptors, nuclear receptors, and protein kinases that are drug targets for depression, breast cancer, and cardiovascular disease (Plassart-Schiess, E. and Baulieu, E. E., *Brain Res Brain Res Rev*, 2001, 37:133-40; Strott, C. A., *Endocr Rev*, 1996, 17:670-97; Kuroki, T., et al., *Mutat Res*, 2000, 462:189-95). Development of more selective therapies for these disorders by pharmaceutical companies is currently hampered by a lack of molecular assays and purified SULT isoforms for high throughput screening (HTS) of potential SULT substrates and inhibitors.

[000104] There are two major classes of SULTs in humans that differ in solubility, size, subcellular distribution, and share less than 20% sequence homology. The membrane bound enzymes are located in the golgi apparatus and sulfonate large endogenous molecules such as heparan, glycosaminoglycans, and protein tyrosines (Strott, C. A., *Endocr Rev*, 2002, 23:703-32). The cytosolic sulfotransferases, or SULTs sulfonate small molecular weight xenobiotics and hormones.

[000105] There are eleven known cytosolic SULT isoforms, which differ in their tissue distribution and specificity. The nomenclature used is based on homology at the amino acid level: SULTs within the same family, indicated by a number, share at least 45% amino acid identity, and those within the same subfamily, designated by a letter, share at least 60% identity. The SULTs can be differentiated to some degree based on substrate specificity, though their substrate profiles overlap extensively, even between enzymes in the different families.

[000106] Structural studies have revealed that there is flexibility in the substrate binding sites and that ligand binding elicits the transition to a more ordered structure,

which may contribute to the broad substrate specificity of the SULTs (Bidwell, L. M., et al., J Mol Biol, 1999, 293:521-30). For this reason, it is not likely that computational approaches such as molecular docking may be applicable for identifying SULT substrates and inhibitors; this increases the value of the HTS assays encompassed by the present invention.

[000107] It is envisioned that the assays of the invention may be used in an HTS format to provide for example, SULT metabolism information such as whether the compound of interest interacts with one or more SULT isoforms, whether the compound is a substrate or inhibitor, and the kinetic parameters (IC_{50} , K_m , V_{max}) for the interaction. The HTS assay encompassed by the present invention provides all of this information by screening in two different modes: a) direct measurement of test compound turnover; and b) measurement of compound inhibition of probe substrate sulfation. In the direct turnover mode, compounds could be screened vs. a panel of SULT isoforms to profile compound sulfation by individual SULT isozymes. After isozyme identification, more detailed kinetic studies with the appropriate SULT isozyme could be used to predict *in vivo* clearance rates (Obach, R. S., et al., J Pharmacol Exp Ther, 1997, 283:46-58), reducing the failure rate of compounds in clinical studies (Greco, G. N., E; Martin, YC, 1998, 219-245). SULT inhibitors could be identified by using a known substrate - e.g. estradiol for SULT1E1 - and screening test compounds for inhibition. Isozyme specific kinetic and inhibition data will further improve drug discovery by providing knowledge of metabolism by specific SULT alerting the drug discovery team to potential drug-drug interactions and pharmacogenetic issues. The contribution of genetic differences in SULTs to varying responses to therapeutics is an active area of investigation (Thomae, B. A., et al., Pharmacogenomics J, 2002, 2:48-56).

[000108] Identification of the SULT responsible for the metabolism of a drug will aid in judicious selection of the *in vitro* assays or animal models used for preclinical assessment of possible drug-drug interactions and toxicology testing, thereby reducing inappropriate or unnecessary experimental animals use. Metabolism data can be used as a component of rational drug design and lead optimization. A better understanding of the structure-activity relationships that define substrate specificity for the various SULT isozymes may provide a basis for structural modifications of primary compounds to change their metabolism profile (Greco, G. N., E; Martin, YC, 1998, 219-245). Accordingly, the methods of the present invention will enable a better understanding of the structure-activity relationships that define substrate specificity for the various SULT isozymes.

Integrated approach to drug discovery

[000109] The ability of the assays of the invention to be used as HTS assays enables a rational, integrated approach to drug development for therapeutic areas where for example, sulfation is a component of the relevant signal transduction biology. Some specific areas where the HTS methods of the invention may be used include for example steroid hormone based therapies. Sulfation, in accordance with the present invention, encompasses involvement in estrogen level regulation in mammary tumors, as well as androgen levels in prostate tumors. Furthermore, cortisol sulfation, though not well understood, inactivates the hormone for binding to the glucocorticoid receptor. High throughput biochemical assays for steroid ligand-receptor binding and the resultant binding of transcriptional coregulator proteins are already commercially available and being used by pharma HTS groups to find more selective steroid hormone modulators (Parker, G. J., et al., J Biomol Screen, 2000, 5:77-88; Spencer, T. A., et al., J Med Chem, 2001, 44:886-97). As such, the availability of robust HTS assays for steroid sulfation may provide an important addition to the arsenal of molecular tools available to pharma groups focused on steroid signal transduction. For instance, the inhibition of SULT1E1 by compounds targeting the ER could cause deleterious effects by elevating estrogen levels in tumor cells. Molecules with SULT1E1 inhibitory properties could be culled or modified based on studies using the assay methods of the present invention.

[000110] The modulation of neurosteroids is being investigated as a novel pharmacological approach to controlling neural excitatory balance (Malayev, A., et al., Br J Pharmacol, 2002, 135:901-9; Maurice, T., et al., Brain Res Brain Res Rev, 2001, 37:116-32; Park-Chung, M., et al., Brain Res, 1999, 830:72-87). The methods encompassed by the present invention may suitably accelerate these efforts by allowing facile screening of endogenous and synthetic neurosteroids for sulfoconjugation, offering insight into the fundamental biology as well as providing a tool for lead molecule identification and optimization. The need for better molecular tools is accentuated by the fact that there is already a sizeable over the counter market for DHEA as an “anti-aging” dietary supplement purported to alleviate age related senility and memory loss (Salek, F. S., et al., J Clin Pharmacol, 2002, 42).

[000111] Furthermore, it is envisioned that the methods of the present invention may suitably identify drug targets with respect to cholesterol sulfate in the regulation of cholesterol efflux, platelet aggregation and skin development in treatments for cardiovascular disease and perhaps some forms of skin cancer. In this instance, a sulfotransferase - most likely SULT2B1b – could become the drug target, and molecules that selectively inhibit this isoform may need to

be identified. The availability of a full panel of the human SULTs and a robust HTS assay method of the present invention may be valuable to such an effort.

Glucuronidation in Drug Discovery

[000112] Drug metabolism problems such as production of toxic metabolites and unfavorable pharmacokinetics cause almost half of all drug candidate failures during clinical trials (Obach, R. S., et al., J Pharmacol Exp Ther, 1997, 283:46-58). All of the major pharmaceutical companies have recognized the need to consider pharmacokinetic and pharmacogenomic consequences early in the drug discovery process resulting in an immediate need for high throughput *in vitro* methods for assessing drug metabolism. Aside from P450-dependent oxidation, glucuronidation is perhaps the most important route of hepatic drug metabolism. A broad spectrum of drugs are eliminated or activated by glucuronidation including non-steroidal anti-inflammatories, opioids, antihistamines, antipsychotics and antidepressants (Meech, R. and Mackenzie, P. I., Clin Exp Pharmacol Physiol, 1997, 24:907-15; Radominska-Pandya, A., et al., Drug Metab Rev, 1999, 31:817-99). Despite their importance, the broad and overlapping substrate specificity of the hepatic UDP-glucuronosyltransferases (UGTs) that catalyze glucuronidation remains poorly understood because of a lack of flexible *in vitro* assay methods. The primary reasons for this is that the catalytic assays used require separation of reactants from products, which involves substrate-specific chromatographic steps and thus is not practical in an HTS format.

[000113] Two UGT families (e.g., UGT1 and UGT2) have been identified in humans; although the members of these families are less than 50% identical in primary amino acid sequence, they exhibit significant overlap in substrate specificity. The members of the UGT1 family that are expressed in human liver, where the majority of xenobiotic metabolism takes place, include UGT 1A1, 1A3, 1A4, 1A6, and 1A9. Although the UGT2 family has not been studied as extensively, it is known that UGT2B4, 2B7, 2B10, 2B11 and 2B15 are expressed in the liver. Mutations in UGTs are known to have deleterious effects, including hyperbilirubinaemia which occurs with a frequency of 5-12% (Weber, W., 1997) and can lead to neurotoxicity and in severe cases, death. As is the case for other drug metabolizing enzymes such as P450s, interindividual differences in UGT expression levels have been observed and linked to differences in drug responses (Iyer, L., et al., J Clin Invest, 1998, 101:847-54). For instance, low expression of UGT1A1, as in patients with Gilbert's syndrome, has been associated with the toxicity of Irinotecan, a promising anticancer agent (Wasserman, E., et al., Ann Oncol, 1997, 8:1049-51). In addition, UGT upregulation in tumor tissues has been

identified as a possible cause of anticancer drug resistance (Franklin, T. J., et al., Cancer Res, 1996, 56:984-7; Takahashi, T., et al., Jpn J Cancer Res, 1997, 88:1211-7).

[000114] All of the known UGTs exhibit broad substrate specificity, with a single isozyme catalyzing glucuronidation of a broad range of structurally unrelated compounds; not surprisingly there also is a great deal of overlap in the specificities of UGT isozymes (Radomska-Pandya, A., et al., Drug Metab Rev, 1999, 31:817-99). With regards to biotransformation of endogenous molecules, UGT 1A1 is clearly the predominant isoform involved in glucuronidation of the tetrapyrrole, bilirubin, resulting in its excretion. Beyond this, it is difficult to make generalizations regarding specificity because of the lack of systematic studies with most of the recently identified isoforms. Numerous endogenous steroids have been identified as aglycones for most of the hepatic isoforms including 1A1, 1A3, 1A4, 2B4, 2B7, and 2B15. Lipids and bile acids serve as substrates for 2B4 and 2B7, and recently retinoids have been identified as substrates for some isoforms from both families. The structural diversity of known xenobiotic aglycones is very broad; it includes many drugs and drug like molecules including tertiary amines such as imipramine, non-steroidal anti-inflammatories (NSAIDs) such as acetaminophen and naproxen, opioids such as morphine and codeine, and carboxylic acid containing drugs such as clofibrilic acid.

[000115] In the short term, pharmaceutical companies have an immediate need for better methods to determine whether their potential drug candidates will be glucuronidated *in vivo*, and if so by which UGT isoform. And in the long term, developing the ability to predict the metabolism of drugs by glucuronidation will require a systematic effort to fully define the “chemical space” recognized by each of the key hepatic UGTs. The proposed Phase II studies will generate the molecular tools required for this effort, including HTS assay methods that can be used to rapidly screen large numbers of diverse chemicals for binding and metabolism by isolated UGT isoforms.

[000116] Pharmaceutical research and development is time consuming, expensive, and inefficient, resulting either in higher costs or lower availability of new therapies for the U.S. health care consumer. Currently, development of a new drug in the USA requires ten to fifteen years and a total R&D outlay of \$400 to \$750 million. While clinical trials are the most expensive phase of development, typically accounting for 30-50% of the total R&D cost, only 10% of all drug candidates tested in clinical trials ultimately are commercialized (Obach, R. S., et al., J Pharmacol Exp Ther, 1997, 283:46-58). Moreover, an analysis of the reasons for drug candidate attrition during clinical trials confirms that

some of the key determinants of the success or failure of a compound are a function of its metabolism, including rate of clearance (pharmacokinetics), potential for interference with the metabolism of other drugs, and potential for generating toxic metabolites. The combination of poor pharmacokinetics--usually excessively rapid clearance--and toxicity cause over 50% of all clinical failures. Gaining a better understanding of how potential drug candidates are metabolized early in the discovery phase would improve the success rate in preclinical and clinical studies resulting in more efficient drug development, and increased economy and availability of therapies.

[000117] It is envisioned that the immunoassay (i.e., FPIA-based donor product assay) for UGTs will be used in a manner very similar to that described for SULTs for determining whether potential drug candidates interact with any of the known UGT isoforms. Using the method of the invention, it will be possible to determine whether compound of interest interacts with a one or more UGT isoforms, and if so, whether it is a substrate or inhibitor. Also one can identify the kinetic parameters (IC_{50} , K_m , V_{max}) for the interaction between the compound of interest and enzymatic isoform. It should be noted that recombinant forms of the many of the UGT isoforms are already available (Invitrogen, Becton-Dickinson). The information obtained with these HTS assays can be used in the following ways such as after isozyme identification, more detailed kinetic studies with the appropriate UGT isozyme can be used to predict *in vivo* clearance rates, reducing the number of compounds that fail in clinical studies due to poor pharmacokinetics. Also, the knowledge of metabolism by a specific UGT alerts the drug discovery team to potential pharmacogenetic problems, since genetic differences in UGT levels are recognized as an important factor in varying responses to therapeutics. Furthermore, the identification of the UGT responsible for the metabolism of a drug will aid in judicious selection of the *in vitro* assays or animal models used for preclinical assessment of possible drug-drug interactions and toxicology testing, thereby reducing inappropriate or unnecessary use of animals for experiments. Also, metabolism data can be used as a component of rational drug design. A better understanding of the structure-activity relationships that define substrate specificity for the various UGT isozymes would provide a basis for structural modifications of primary compounds to change their metabolism profile. Also, the testing of glucuronidated compounds can lead to the discovery of valuable prodrugs that are inactive until metabolized in the body into an active form.

Protein Kinases in Drug Discovery

[000118] There are more than 400 distinct kinases encoded in the human genome; elucidating their role in disease and identifying selective inhibitors is a major pharma initiative. Kinase malfunction has been linked to all of the most important therapeutic areas, including cancer, cardiovascular diseases, inflammation, neurodegenerative diseases, and metabolic disorders. Moreover, clinical validation of kinases as drug targets has recently been shown in the cases of Herceptin and Gleevec, which inhibit aberrant tyrosine kinases that contribute to breast cancer and leukemia, respectively. High throughput screening (HTS) – the parallel testing of many thousands of compounds for interaction with a drug target - has become the dominant mode of drug discovery. The total market for HTS assay reagents in 2002 was \$474M, and approximately 20% of screening was done on protein kinases. Despite the high level of interest, pharma efforts to incorporate kinases into HTS programs are hampered by shortcomings with the assay methods. The most commonly used HTS kinase assays rely on fluorescence-based immunodetection of a phosphorylated peptide reaction product, which varies with the substrate specificity of individual kinases. Time consuming reagent development is thus required for each kinase, or group of related kinases, and comparison of results between assays is problematic. To overcome this technical hurdle the invention provides for a FPIA for detection of adenosine diphosphate (ADP), a product of all kinase reactions. This assay will accelerate efforts to define kinase substrate specificity and to identify novel inhibitors by providing a universal catalytic assay that can be used with any kinase and any acceptor substrate.

[000119] Protein Kinases are a large, diverse family with a key role in signal transduction. Protein kinases, which catalyze the transfer of the terminal phosphate group from ATP or GTP to serine, threonine or tyrosine residues of acceptor proteins, are one of the largest protein families in the human genome. In the broadest senses, they can be divided into serine/threonine or tyrosine kinases and soluble enzymes or transmembrane receptors. In the most recent and comprehensive genomic analysis, 428 human kinases were identified that comprise eight different homology groups, which also reflect differences in substrate specificity, structure/localization and/or mode of regulation (Hanks, S. K., *Genome Biol*, 2003, 4:111). For instance, there are 84 members of the Tyrosine Kinase group, which includes both transmembrane growth factor receptors such as EGFR and PDGFR and soluble enzymes such as the Src kinases, 61 members of the cyclic nucleotide dependent group, ser/thr kinases which includes the lipid dependent kinases – the PKC isoforms, and 45 members of the “STE” group, which includes the components of the mitogenic MAP kinase signaling pathway.

[000120] Kinases are ubiquitous regulators of intracellular signal transduction pathways, and as such have come under intense focus by pharmaceutical companies searching for more selective therapies for a broad range of diseases and disorders; they are second only to G-protein coupled receptors in terms of pharma prioritization (Cohen, P., Nat Rev Drug Discov, 2002, 1:309-15). Intracellular targets for phosphorylation include other kinases, transcription factors, structural proteins such as actin and tubulin, enzymes involved in DNA replication and transcription, and protein translation, and metabolic enzymes (Cohen, P., Trends Biochem Sci, 2000, 25:596-601). Phosphorylation can cause changes in protein catalytic activity, specificity, stability, localization and association with other biomolecules. Simultaneous phosphorylation at multiple sites on a protein, with different functional consequences, is common and central to the integration of signaling pathways.

Diversity of Phosphorylation Sites.

[000121] Each kinase may phosphorylate one or more target proteins, sometimes at multiple sites, as well as autophosphorylate within one or more regulatory domains that control catalytic activity or interaction with other biomolecules. Defining the functional consequences of cellular phosphorylation profiles for normal and disease states is a major proteomics initiative. However, to use this knowledge for deciding which kinases to target for drug discovery, their specificity for acceptor substrates must also be delineated. Kinases recognize specific linear sequences of their target proteins that often occur at beta bends. In general, amino acids that flank the phosphorylated residue for three to five residues on either side define a phosphorylation site. The PhosphoBase database, which compiles known kinase phosphorylation sites, contains entries for 133 human kinases, less than a third of the total kinases. Moreover, most, if not all of these specificity profiles are incomplete, as they only show one or two peptides that have been identified as substrates for each kinase. Though there is significant overlap in substrate specificity among related kinases, there is no consensus sequence that is phosphorylated by a large number of kinases. This situation complicates the incorporation of diverse or novel kinases into HTS assays that rely on detection of specific phosphorylated products.

TABLE 1

Target	Structure: Company (Phase)
EGFR	Small molecule: GlaxoSmithKline (I), Pfizer (II), WyethAyerst (I), AstraZeneca (III), OSI Pharmaceuticals
	Monoclonal: Imclone (III), Abgenix (II), Merck (I), Medarex/Merck (I)
Her2/Neu	Monoclonal: Genentech (Herceptin), Medarex/Novartis (I), Genentech (I)
PDGFR/cKit/BCR-Abl	Small molecule: Novartis (Gleevec)
Raf	Small Molecule: Merck, Onyx/Bayer (II)
	Antisense: Isis Pharmaceuticals (II)
MEK	Small molecule: Pfizer (II), Promega (I)
CDK	Small molecule: Aventis (II), Cyclacel (I), Bristol-Myers Squibb (I)
PKC	Small molecule: Novartis (II), GPC Biotech (II), Eli Lilly (I)
	Antisense: Isis (III)

Table 1. Selected clinical trials for development of kinase inhibitors as anticancer agents (Dancey, J. and Sausville, E. A., Nat Rev Drug Discov, 2003, 2:296-313). Bolded drugs are approved

Protein Kinases in Cancer

[000122] The biological rationale for targeting kinases to intervene in cancer is far too extensive to attempt an overview here. However, one of the dominant themes is the involvement of numerous kinases in controlling the delicate balance between the rate of cell division (cell cycle progression), cell growth (mass), and programmed cell death (apoptosis) that is perturbed in all cancers. Growth factor receptor tyrosine kinases (RTKs) are membrane-spanning proteins that transduce peptide growth factor signals from outside the cell to intracellular pathways that lead to activation of progrowth and anti-apoptotic genes. The majority of the fifty-eight RTKs in humans are dominant oncogenes, meaning that aberrant activation or overexpression causes a malignant cell phenotype. Not surprisingly, tyrosine kinases are being aggressively pursued as anticancer drug targets and both small molecule and monoclonal antibody inhibitors – Gleevec and Herceptin, respectively - have been clinically approved. Downstream signaling from growth factor receptors occurs through multiple pathways involving both ser/thr and tyrosine kinases. One of the dominant kinases is the mitogen activated protein kinase (MAPK) pathway, which includes Raf and MEK kinases; inhibitors of all of these kinases are currently being tested in clinical trials (Table 1) (Dancey, J. and Sausville, E. A., Nat Rev Drug Discov, 2003, 2:296-313). Soluble tyrosine kinases, especially the 11 oncogenes that comprise the Src family, also transduce mitogenic signals initiated by RTKs and are being targeted by pharma (Warmuth, M., et al., Curr Pharm Des, 2003, 9:2043-59). Following mitogenic signals through RTKs that initiate entry into the G1

phase, progression through the cell cycle is regulated by sequential activation of phase-specific kinases in association with cyclin proteins. The cyclin dependent kinases represent yet another important group of kinases that pharma is pursuing in the hopes of inhibiting malignant cell proliferation (Table 1) (Elsayed, Y. A. and Sausville, E. A., *Oncologist*, 2001, 6:517-37).

Kinases as Targets in Other Diseases

Pharma interest in kinases is most intensely focused on cancer, but extends to all of the key therapeutic areas. Table 2 shows recent reviews describing the biological rationale for pursuing kinases targets for a broad range of disorders; note that the targets overlap those being pursued for cancer. Entire companies have formed on the basis of targeting kinases for drug discovery. These include Sugen (<http://www.sugen.com>, now owned by Pfizer), Signase (<http://www.signase.com/index.htm>), and ProQinase (<http://www.proqinase.com/index.html>).

TABLE 2

Disease	Targets
Inflammatory diseases, arthritis	MAPK (Adams, J. L., et al., <i>Prog Med Chem</i> , 2001, 38:1-60); MEK1,2 (English, J. M. and Cobb, M. H., <i>Trends Pharmacol Sci</i> , 2002, 23:40-5)
Type II diabetes and complications	GSK-3 (Eldar-Finkelman, H., <i>Trends Mol Med</i> , 2002, 8:126-32), PI-3 Kinase (Jiang, G. and Zhang, B. B., <i>Front Biosci</i> , 2002, 7:d903-7), PKC β (Frank, R. N., <i>Am J Ophthalmol</i> , 2002, 133:693-8), IRTK (Laborde, E. and Mancham, V. P., <i>Curr Med Chem</i> , 2002, 9:2231-42), PKA (Musi, N. and Goodyear, L. J., <i>Curr Drug Targets Immune Endocr Metabol Disord</i> , 2002, 2:119-27)
Hypertension, cardiovascular	PKC, var. isoforms (Malhotra, A., et al., <i>Mol Cell Biochem</i> , 2001, 225:97-107), Rho Kinase (Chitaley, K., et al., <i>Curr Hypertens Rep</i> , 2001, 3:139-44)
Neuropathy - Alzheimer's disease, stroke	CDK5 (Lau, L. F., et al., <i>J Mol Neurosci</i> , 2002, 19:267-73); MAPK (Dalrymple, S. A., <i>J Mol Neurosci</i> , 2002, 19:295-9), CKDs (O'Hare, M., et al., <i>Pharmacol Ther</i> , 2002, 93:135-43)
Neuropsychiatric disorders	PKC α , PKC ϵ (Chen, G., et al., <i>Bipolar Disord</i> , 2000, 2:217-36)
Antimicrobials	Histidine Kinases (Matsushita, M. and Janda, K. D., <i>Bioorg Med Chem</i> , 2002, 10:855-67)

Table 2. Listing of links between protein kinases and various diseases.

[000123] It is envisioned that the FPIA-based donor product assay will be used to screen drug libraries for inhibitors or activators of protein kinases. It will also be useful for screening peptides or proteins as acceptor substrates for kinases. In these applications, it will have the significant advantages over other methods such as the universal nature of the assay, simplified homogenous assay, no radioactivity, and the ability to quantify enzyme turnover.

Universal Assay Method

[000124] This method will accelerate the incorporation of protein kinases into HTS screening programs because it is truly generic: a single set of detection reagents can be used for all kinases and all acceptor substrates. An important advantage over most of the current assay methods is the capability to use physiological protein acceptor substrates as well as short peptides.

Homogenous

[000125] This assay is a single addition, mix and read format. This is an important factor driving decisions on assay selection in an automated high throughput environment (High Tech Business Decisions, M., CA, Commissioned Market Analysis, 2002). In addition, if antibodies with suitable binding kinetics are isolated, it allows a continuous assay format that provides more detailed kinetic information than a stop-time assay.

Fluorescence detection

[000126] By employing fluorescent probes, the FPIA format eliminates radiation handling, disposal and costs. It should be noted that over the last few years FP has become one of the key HTS assay platforms used by pharma (Owicki, J. C., J Biomol Screen, 2000, 5:297-306). It is expected that in 2003 it will be used by pharma in approximately 12% of total primary screening assays; this is a doubling from the level of FP usage in 2001 (High Tech Business Decisions, M., CA, Commissioned Market Analysis, 2002). FP is a standard mode on several commercial HTS plate readers.

Quantitative

[000127] In the proposed enzymatic assay, the ADP is produced in stoichiometric amounts with the phosphorylated peptide or protein, thus the use of a standard curve for ADP will allow a direct measure of enzyme turnover. Though the use of FP for HTS applications is a relatively

recent development, the use of FPIAs for quantitative detection of hormones and metabolites in a diagnostic setting is very well established (Nasir, M. S. and Jolley, M. E., Comb Chem High Throughput Screen, 1999, 2:177-90).

EXAMPLES

Example 1. Uridine Glucuronide Transfersase Assay

[000128] One embodiment of this application involves detecting the “donor product” of the UGT reaction using a competitive fluorescence polarization immunoassay where the antibody-bound tracer has a high polarization value which decreases when it is displaced by an analyte, such as UDP (as shown in Figure 2). The main reagent required for this assay is the production of an antibody that binds UDP with high selectivity and has negligible binding to the donor, UDP-glucuronic acid (UDPGA). This highly selective antibody is used in combination with a commercially available fluorescent UTP compound to establish the assay.

[000129] The polyclonal antibody produced against UDP required covalent binding to a carrier protein. UTP was used as the hapten only because reactive derivatives of the triphosphate, but not the diphosphate, were readily available that could be used for conjugation. It was reasoned that the majority of the triphosphate may be hydrolyzed to di- and mono-phosphate in animals. Several different chemistries for linking the UTP to carrier protein were investigated, because the nature of the linkage can have a profound affect on the resulting antibody specificity and affinity for antigen. Care was taken so that the linker molecule was attached to the uridine ring rather than the ribose or phosphate, thus maximizing the immunoreactivity with the portion of the UDP molecule that may differentiate it from the donor, UDPGA.

[000130] Rabbit antiserum raised against a mixture of UTP and UDP conjugated to BSA and a commercially available tracer molecule, a fluorescently labeled UTP compound (Alexa-UTP, Molecular Probes) was added to wells of a black multiwell plate (Thermo Labsystems Pt#7605) containing the indicated amounts of uridine nucleotides. Alexa-UTP was used as a tracer for the FPIA experiments. Fluorescence polarization was read in a Tecan Ultra plate reader after several hours of equilibration. Reaction conditions were as follows: 50 mM KPO₄ pH 7.4, 150 mM NaCl, 0.1 mg/ml BGG, 1 nM ChromaTide Alexa Fluor 488-5-UTP, 1.25 ul rabbit sera, 100µl total volume.

[000131] The experimental results from the UGT reaction are provided in Figures 8A-B. Figure 8 shows titrations of antibody-tracer complex with various uridine nucleotides using the first polyclonal antibody raised against UDP/UTP and a commercially available tracer molecule

(Alexa-UTP). It is noted that the two graphs differ in the scale of the X-axis and that competition by UDP, the donor product is half maximal at approximately $10\mu\text{M}$, whereas for UDPGA, the donor, half maximal displacement is higher than 1mM which is at least a 100x difference in selectivity.

[000132] Most relevantly, whereas UDP displaces the tracer at low micromolar concentration, there is no detectable crossreactivity with UDPGA at concentrations in excess of $100\mu\text{M}$. The crossreactivity with UTP is not problematic for the proposed assay because it is not present, nor is it produced, in UGT enzyme reactions. The ability of UDP to displace the tracer at low micromolar concentrations means that this antibody is suitable for detection of UDP produced in UGT enzyme reactions. Furthermore, since, through preliminary binding kinetic studies there have been indications that the displacement of tracer by UDP is very fast, use of antibody for monitoring UGT turnover in real time; i.e., a continuous assay, is encompassed within the invention.

[000133] Despite their importance, the broad and overlapping substrate specificity of the hepatic UDP UGTs that catalyze glucuronidation remains poorly understood because of a lack of flexible *in vitro* assay methods. The primary problem is that the catalytic assays used require separation of reactants from products, which involves substrate-specific chromatographic steps and thus is not practical in an HTS format. By establishing the concept for measuring the UGT reaction product, UDP in a fluorescent, homogenous format, the applicants have provided the technical foundation for solving this problem. Because the assay measures a product common to all UGT reactions, it allows a single mode of detection with any UGT isoform and any substrate. In addition, the method does not require separation of reactants from products, and is a significant improvement over other related assays because it uses fluorescence detection rather than colorimetric, making it more sensitive and more desirable for pharma HTS platforms, which have become largely reliant on fluorescence based detection. Also, it is a continuous assay method, thus can provide real time kinetic data on UGT enzyme turnover. In addition the antibody-antigen binding reaction is less susceptible to interference from test samples than a conventional coupled enzyme reaction that has been used in the past for donor product detection (Mulder, G.J. and A.B.D. Van Doorn, Biochem J., 1975, 151: p. 131-40). Thus, the novel assay method will enable screening of diverse compounds for metabolism by a panel of isolated UGT isozymes, which will greatly enhance preclinical metabolism studies, and potentially reduce the clinical attrition rate.

[000134] Furthermore, the properties of the antibody produced by the applicants have broader implications for development of HTS assays for other important classes of enzymes. As

shown in Figure 8, UDP, but not UMP displaces the tracer, illustrating that the antibody is capable of differentiating between nucleotides on the basis of a single phosphate group. Thus, it is encompassed that antibodies against other “donor products” such as ADP for protein kinase reactions where the reaction product differs from the donor by a single phosphate may be produced.

Example 2. Kinase Assays

[000135] The assay method of the invention relies on FPIA detection of a kinase reaction product ADP that is produced in stoichiometric amounts with phosphorylated polypeptide. Similar to the UGT assay described above, the components of the FPIA based donor product kinase assay include an antibody to the donor product and a tracer comprised of a donor product conjugated with a detectable tag. The antibody is highly specific for ADP (i.e., it is capable of recognizing ADP in the presence of excess ATP and a fluorescent tracer). The antibody and tracer in a kinase assay are added to wells of a black multiwell plate at concentrations optimal for the start of the assay. A suitable concentration of tracer is 1- 2nM and sufficient antibody is used to cause approximately 75% of the maximal polarization shift for the tracer. The acceptor substrate is added to the wells at the desired concentration, generally 2-5-fold higher than the K_m value. The acceptor substrate can be a peptide or an intact protein. One benefit of the invention is that any acceptor substrate can be used, whereas other kinase assay methods require a specific acceptor for detection. A buffer that is compatible with the kinase to be assayed and the antibody-tracer interaction, generally a Tris-Cl or phosphate buffer at about a neutral pH is used. With the buffer, the other required components of the assay are added, including ATP at a concentration of 100 μ M-5mM, $MgCl_2$, and any other agents required for activation or stabilization of the kinase. The kinase enzyme is then added to initiate the reaction and the polarization values are monitored in a multiwell reader such as the Tecan Ultra. As the reaction proceeds, ADP produced in stoichiometric amounts with the phosphorylated peptide or protein displaces the tracer from the antibody resulting in decreased polarization values.

[000136] In screening for inhibitors, the compounds to be tested are generally dispensed into wells prior to addition of any other assay components, and control wells with no inhibitor added are included for comparison.

[000137] Each kinase may phosphorylate one or more target proteins, sometimes at multiple sites, as well as autophosphorylate within one or more regulatory domains that control catalytic activity or interaction with other biomolecules (Cohen, P., Trends Biochem Sci, 2000, 25:596-601). Defining the functional consequences of cellular phosphorylation profiles for

normal and disease states is a major proteomics initiative, and this knowledge can be used for deciding which kinases to target and their specificity for acceptor substrates in drug discovery. Though there is significant overlap in substrate specificity among related kinases, there is no consensus sequence that is phosphorylated by a large number of kinases. This situation complicates the incorporation of diverse or novel kinases into HTS assays that rely on detection of specific phosphorylated products.

Example 3. Sulfotransferase Assays

Expression and Purification of SULT1E1

[000138] In order to establish a sulfotransferase HTS assay method, SULT1E1, a SULT isoform, was first subcloned into an E. coli expression vector with a C-terminal 6x histidine tag and the expressed protein was purified by affinity chromatography and characterized with respect to its physical and enzymatic properties.

[000139] The purified protein migrated close to its calculated molecular weight on SDS-PAGE and more importantly, mass spectral analysis agreed very closely with predicted molecular weight (36,161 and 36,374 daltons, respectively). Because it was designed to retain a native N-terminus, the purified C-terminal 6xHis construct was also subject to N-terminal sequencing; 15 amino acids were sequenced and they were identical with the human SULT1E1 sequence in GenBank (NP005411). Further sequence verification was obtained through mass spectral analysis and protein sequencing.

[000140] To serve as a comparison, the enzymatic properties of the purified SULT1E1 were examined. Estradiol and estrone were used as physiological substrates and additional positive (α -naphthol) and negative (dopamine) control compounds tested to assess specificity. Two types of radioassays were used for these studies, one using ^{35}S -PAPS (Foldes, A. and Meek, J. L., Biochim Biophys Acta, 1973, 327:365-74), and the other using ^3H -estradiol (Zhang, H., et al., J Biol Chem, 1998, 273:10888-92); both have been used extensively for SULT characterization and are described below.

TABLE 4

Construct	Kinetic Parameters w/ Estradiol			Relative Reaction Rates with Various acceptors				
	K_m (nM)	V_{max} nmol/min/ mg	V_{max}/K_m	Estradiol	Estrone	α - naphthol	DHEA	Dopa- mine
hSULT1E1- 6xHis	15	130	8.67	100%	39%	41%	11%	2%

[000141] Table 4 shows the enzymatic properties of SULT1E1 fusion proteins. K_m and V_{max} determinations were done using 3H -estradiol radioassays in which the radioactive sulfated product is separated from unreacted 3H -estradiol by organic/aqueous phase extraction and counted in a liquid scintillation counter. Kinetic parameters were calculated from V vs. S curves by nonlinear regression using GraphPad/Prizm software. The relative reaction rates with various acceptors were determined using 400nM acceptor substrate in the ^{35}S -PAPS radioassay, in which the unreacted ^{35}S -PAPS is precipitated as a barium-metal complex and the supernatant containing the ^{35}S -labeled sulfoconjugate is counted in a liquid scintillation counter. Basal reaction conditions in both cases were 10 mM KPO_4 pH 6.5, 10 mM DTT, 1.5 mM $MgCl_2$, 10 mM PAPS, 0.5 ng SULT 1E1, 0.0025 to 15 mM acceptor.

[000142] The V_{max} and estradiol K_m values determined for the purified SULT1E compared favorably with published values for purified recombinant SULT1E1, which are 30-40nmol/min/mg and 5-15nM, respectively. The published data on acceptor substrate specificity is more varied, but the results reflect the general trend that estradiol and estrone are very good substrates, α -naphthol is intermediate, and DHEA and dopamine are very poor substrates (see Table 4). Thus all of the SULT1E1 constructs applicants expressed showed native substrate specificity and catalytic rates similar to the highest published values.

Synthesis of antigens and generation of antibodies

[000143] As to the actual development of the fluorescence-based HTS assay for sulfotransferases, there were four components showing a PAP FPIA that detected SULT catalysis: synthesis of antigens and generation of antibodies; synthesis of tracer molecules; testing antibodies and tracers for interaction and specificity; and demonstrating detection of SULT1E1 activity using optimal antibody-tracer pairs. Tracer synthesis efforts overlapped significantly with antigen synthesis efforts, as the same reactive PAP derivatives were used for conjugating to carrier protein and fluorophores.

[000144] Development of the proposed FPIA-based SULT assay method requires an antibody that specifically binds the product of the SULT reaction, PAP, in the presence of excess PAPS; i.e., an antibody that discriminates on the basis of a single 5'-sulfate group. There is ample precedent for antibodies that discriminate between various nucleotides that differ only in the number of phosphates, which is similar in size and structure to a sulfate

group as described above. However, there was no precedent for generation of antibodies that specifically recognize PAP.

[000145] Small molecules like PAP must be conjugated to a carrier protein in order to be used as an immunogen. Suitably, an antigen density of 10-20 per carrier protein is optimal. As discussed above, the two elements of our antigen synthesis strategy were a) synthesis and testing of several antigens because the site of attachment to nucleotide and linker structure can profoundly affect the properties of the resulting antibodies (Crabbe, P., et al., J Agric Food Chem, 2000, 48:3633-8; Signorini, N., et al., Chem Res Toxicol, 1998, 11:1169-75; Oda, M. and Azuma, T., Mol Immunol, 2000, 37:1111-22), and b) conjugation via the adenine ring because this may allow free exposure of the 5-phosphate group and limit immunoreactivity with the adenine portion of the molecule, resulting in antigens with the desired specificity. None of the nitrogens in the adenine ring were reactive enough to conjugate directly to protein or via crosslinking reagents, so reactive PAP derivatives were used as a starting point; the only commercially available reagent was N6-aminohexyl PAP. What initially appeared to be the most straightforward approach - reacting N6-aminohexyl-PAP with carrier proteins by carbodiimide coupling or amine-reactive crosslinkers - generated unacceptably low antigen density, despite a significant experimental effort.

[000146] To pursue alternative chemistries applicants outsourced the synthesis of the photoreactive molecules, 2- and 8- azido-PAP and C8-hexylamino-PAP (Figure 6). Figure 6 shows the synthesis of PAP antigens. 2- and 8-azido-PAP, which were custom synthesized by ALT, Inc. (Lexington, KY) were irradiated (254nm) in the presence of BSA; unreacted nucleotides were removed by filtration and dialysis. Final antigen densities of 7-12 PAP/BSA were obtained as determined by absorbance of the adenine ring (shifted to 270-280nm). Rabbits (3 per antigen) were immunized by Lampire Biologicals (Ottsville, PA). N6-aminohexyl PAP (Sigma) was conjugated to KLH using glutaraldehyde and injected into three rabbits by Biosynthesis Corp. (Lewisville, TX). Immunization schedules were similar for all antigens and included 3-4 injections over a 4-6 week period.

[000147] Antigens from the two photoreactive PAP derivatives were produced and sent out for antibody production; generation of antigen from N6-aminohexyl PAP using glutaraldehyde crosslinking to KLH and production of antibody was contracted out to a separate company (Figure 6). All three antibodies were reported by the contract producers to bind PAP as determined by ELISA (data not shown).

[000148] In addition, applicants synthesized the reactive derivatives N6-carboxymethyl PAP and 2'-O-succinyl-PAP (Figure 7); antibodies from these derivatives have not yet been produced. A detailed description of Ab binding properties using competitive FPIA is provided below following the description of tracer synthesis.

Synthesis of PAP-fluor Tracers

[000149] An FPIA tracer molecule can be divided into three different structural components: the antigen, the fluor, and the linker used to join them; an additional key structural variable is the site of attachment of the linker to the antigen. Because identification of a tracer is largely empirical, applicants used a variety of linkers to join PAP and fluorescein via different sites on each molecule; in most cases the final linker region is a composite of the reactive fluorescein and PAP molecules used.

[000150] The antibody strategy was to conjugate through the adenine moiety in order to generate antibodies that bind specifically to the ribosyl-phosphate group of PAP. The same sites are the obvious sites for fluor conjugation as well in order to leave the desired immunoreactive portion of the tracer molecule free to bind Ab. Also, fluorescein was the preferred fluor used for conjugation. Though red-shifted fluors such as rhodamine are more desirable for HTS applications, development of FP tracers with fluorescein is usually more straightforward because it is less prone to non-specific binding effects and there are numerous activated derivatives available. As to the linker molecule, it affects tracer characteristics in a number of important ways that impact both its antigenic and fluorescence properties. There is generally a balance that must be struck between separating the antigen from the fluor enough to allow unhindered interaction with antibody without creating too much freedom of motion for the fluor. The former can result in lowered affinity Ab binding and in quenching of the fluor, whereas the latter reduces the polarization shift upon Ab binding, thereby reducing the dynamic range of the assay.

[000151] N6-aminoethyl PAP was the only activated PAP molecule used for immunogen synthesis that was useful for tracer synthesis; the photoactivation reactions required to conjugate the two azido-PAP derivatives may be inefficient for joining two small molecules. To provide PAP molecules activated at different positions and with different linker regions, applicants outsourced the production of C8-aminoethyl-PAP and synthesized in house two PAP derivatives with carboxy-terminal linkers: N6-carboxymethyl-PAP and 2'-O-succinyl-PAP. Though the latter compound is linked through the ribose hydroxyl rather than the adenine, this approach has been used to

generate highly specific antibodies and tracers for cAMP (Horton, J. K., et al., J Immunol Methods, 1992, 155:31-40).

[000152] Below are brief descriptions of the synthesis and purification of activated PAP molecules and their conjugation to various activated fluors; tracer synthesis components are summarized in Figures 7 and 8. In general, PAP molecules with amino-terminal linkers were conjugated to fluors activated with succinimidyl esters (or isocyanate in one case) and PAP molecules with carboxy-terminal linkers were reacted with fluorescein derivatives containing free amino groups using carbodiimide coupling.

[000153] Figure 7 illustrates structures of components of Tracer Synthesis. Figure 7 from left to right provides PAP molecules with amino-terminal linkers attached at the C8 and N6 position, amine- and carboxy-reactive fluorescein derivatives, and PAP molecules with carboxy-terminal linkers at the N6 and 2'-OH. The fluorescent PAP conjugates were separated by TLC and tested for binding to anti-PAP antibodies. In accordance to the invention, sixteen reactions were run containing different combinations of PAP and fluorescein derivatives and each reaction yielded 1-4 fluorescent products that could be resolved by TLC. In all, more than 40 tracers have been purified and tested for binding to Ab. N6-aminohexyl PAP (Sigma) and all of the reactive fluorescein derivatives (Molecular Probes) are commercially available. 2'-O-succinyl-PAP and N6-carboxymethyl-PAP were synthesized as described below; C8-aminohexyl PAP was synthesized by Jena Biosciences (Jena, Germany). Figure 8 provides representative final tracer structures.

Preparation of N6-carboxymethyl PAP

[000154] To prepare N6-carboxymethyl PAP, 100 mg PAP was incubated with 0.3 g Iodoacetic acid in 1.2 mL aqueous, adjusted to pH 6.5 with LiOH. The reaction proceeded at 30°C for 5-7 days, periodically adjusting the pH to 6.5. The resulting 1-carboxymethyl-PAP product was precipitated with ethanol and reconstituted in distilled water and the pH was adjusted to 8.5 with LiOH. This reaction was heated at 90°C for 1.5 hours to yield the N6-carboxymethyl PAP. This product was purified on a Dowex1-X2 (200-400 mesh) column equilibrated in 0.3 M LiCl, pH 2.75. A gradient was applied over 10 column volumes using 0.5M LiCl, pH 2.0. N6-carboxymethyl PAP eluted off the column as pure product and was confirmed by mass spectral analysis, ~ 20% yield.

Preparation of 2'-O-Succinyl-PAP

[000155] To prepare 2'-O-Succinyl-PAP, 10 mg (0.024 mmole) PAP and succinic anhydride (43 mg, 0.426 mmole) were dissolved in distilled H₂O containing 10% triethylamine (v/v, 1 mL) and shaken for 1.5 hours with reaction progress monitored using reverse phase thin layer chromatography (RP-TLC). Upon completion, the reaction was lyophilized twice to ensure no residual triethylamine remained. The expected succinate was column purified using Fast Flow Sepharose-Q resin (Pharmacia) equilibrated with 50 mL 250 mM NH₄OAc, and eluted with a linear gradient of 500 mM to 750 mM NH₄OAc (50 mL of each). Fractions containing the product were concentrated on a rotovap followed by lyophilization to furnish 8.4 mg or 67% yield as determined by absorbance measurement. Stock solution was stored at -20°C for future use.

Preparation of fluorescein conjugates with amino-activated PAP

[000156] To prepare fluorescein conjugates with amino-activated PAP, 10 µL of a 100 mM solution of N6- or C8-aminoethyl-PAP in distilled H₂O was combined in a screw cap vial with a molar equivalent of a fluorescein succinimidyl ester, the reaction was brought to a final volume of 200 µL using anhydrous dimethyl sulfoxide (DMSO), and shaken on a vortexer for 24 hours. Reaction progress was followed by RP-TLC using H₂O as the developing media. Reaction products were purified using preparative TLC on normal phase silica gel using 1:1 EtOH/0.5 M NH₄OAc. Fluorescein labeled compounds – generally 3-5 produced in each reaction – were visualized using UV light, scraped from the TLC plates, and extracted from the silica gel using 1:1 MeOH/0.5 M NH₄OAc. Individual fractions were shaken on a vortex for 1 hour wrapped in aluminum foil and centrifuged at 4000 RPM for 8 minutes. The supernatants were triturated to a separate labeled vial and the extraction process repeated. Combined supernatants were standardized to 50 nM solution for future use and frozen in amber microfuge tubes at -20°C.

Preparation of fluorescein conjugates with carboxy-activated PAP

[000157] To prepare fluorescein conjugates with carboxy-activated PAP, 10ml of a 100 mM solution of N6-carboxymethyl-PAP or 2'-O-succinyl-PAP in distilled H₂O was combined with 50 equivalents of 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDC) (10 µL of a 1M solution in anhydrous DMSO) followed by 75 equivalents of N-hydroxysuccinimide (15 µL of a 1000 mM solution in anhydrous DMSO), the reaction was brought to an intermediate volume of 180 µL with anhydrous DMSO and shaken for 48

hours. One molar equivalent (20ml) of fluorescein derivative with a free primary amine was then added and the reaction was incubated with vortexing for 24 hr. Reaction progress was followed by RP-TLC using H₂O as the developing media. Upon completion of the reaction, fluorescent products were purified by normal phase silica TLC and eluted as described above, except that 9:1 acetonitrile/2mM NH₄OAc, pH 5.5 was used as the TLC solvent and 8:2 acetonitrile/2mM NH₄OAc, pH 5.5 was used for elution.

[000158] A total of 16 unique combinations of activated PAP and fluorescein molecules were reacted and from these, more than 40 fluorescent products were isolated and tested for binding to antibodies. This approach can be viewed as sort of a “poor man’s combinatorial chemistry,” applicants have found it to be very successful for development of FP tracers in other instances. It is noted that the homogenous nature of FP assays and the availability of multiwell instruments makes the screening efforts relatively rapid.

Characterization of anti-PAP antibodies and tracers

[000159] Analysis of antibody/tracer interaction was done by fluorescence polarization. To measure fluorescence polarization (described briefly above), the emission intensity is measured in both the vertical and horizontal planes, which are parallel and perpendicular to the vertically polarized excitation light. The emission light defines polarization: $P = (\text{horizontal intensity} - \text{vertical intensity}) / (\text{horizontal intensity} + \text{vertical intensity})$ Polarization values are reported as mP, which is 1000 times the polarization value (30 mP = 0.03 P). Fluorescein has a polarization of approximately 20 mP at 37° C in phosphate buffer at pH 7.0. Mathematically, the limiting polarization for fluorescein is 500 mP; practically, an anti-fluorescein antibody bound to fluorescein gives a polarization of ~450 mP. The currently available multiwell FP readers such as the Tecan Ultra can read polarization values with great precision; i.e., detection of 1 nM fluorescein in the free and the bound state with less than <3mP standard deviation.

[000160] In a competitive FPIA, a fluorescently labeled antigen, a tracer, is displaced from binding to antibody by the analyte. The signal is proportional to the difference in the bound versus free tracer fractions, thus both the dynamic range and the sensitivity of the assay are dependent upon the affinity of the antibody for the tracer and the competing sample molecule. To establish a suitable dynamic range for an FPIA, at least 70-80% of the tracer must be bound to antibody in the absence of competitor. This normally is achieved by using the tracer at a concentration 2-10 fold below the K_d and the antibody at a concentration 2-3-fold over the K_d. The useful concentration range of the assay then

ranges from several-fold below the K_d concentration to about 20-fold over the K_d concentration. For example, if the K_d for the tracer/antibody complex is 10 nM, then the range of detection for the sample may be from about 2 nM to about 200 nM. If the K_d is ten fold less (1 nM), the sensitivity of the assay also improves about 10-fold.

[000161] Acceptable properties for anti-PAP antibody and tracer in terms of the assay parameters affected are defined as 1) dynamic range: low tracer polarization in the absence of Ab (<100mP) and maximal difference in polarization between the bound and free states (Δ mP >100mP); 2) sensitivity: high affinity binding of tracer to the anti-PAP antibodies (K_d < 100nM), displacement by free PAP with a similar IC_{50} , and minimal fluorescence quenching caused by binding; 3) signal/noise: high Ab selectivity; i.e., competitive displacement of tracer by PAP and not by PAPS or other adenine nucleotides; also lack of tracer interaction with SULT1E1 or other assay component; and 4) a continuous assay: rapid association/dissociation kinetics.

Results of Ab-tracer interaction studies

[000162] Initial screens for Ab-tracer binding were done by adding several concentrations of each antibody to 1nM tracer in multiwell plates and monitoring for increases in tracer polarization. With all of the antibodies, purification of IgG fractions using immobilized Protein A or Protein G was required to remove non-specific binding components. All of the data shown is using antibodies purified on Protein G (Pierce NabTM Protein G Spin Chromatography Kit). Tracer/Ab combinations that showed an interaction in the initial screen were analyzed in more detail for competitive displacement by PAP and selectivity of PAP over PAPS. A total of nine purified antibodies (3 antigens x 3 rabbits) were tested for binding with 44 different fluorescein-labeled PAP tracers. All assays were done in black 96-well plates or black low-volume 384-well plates, which gave essentially identical results, and polarization values were read on a Tecan Ultra with a fluorescein filter set (excitation at 485 nm to emission at 535 nm) (Ex₄₈₅/Em₅₃₅).

[000163] The binding isotherms in Figure 9 were generated using the three antibodies and two tracers shown in Table 5 below, and are representative of experiments used to screen antibodies and tracers for binding.

TABLE 5. Representative antibodies and tracers

Abbreviation	Description
Ab 1781	8-azido-PAP-BSA immunogen
Ab 1810	2-azido-PAP-BSA immunogen
Ab 3642	N6-aminohexyl-PAP-KLH immunogen
N6-PAP-F8	N6-aminohexyl-PAP conjugated to 6-carboxyfluorescein succinimidyl ester
C8-PAP-F14	C8-aminohexyl-PAP conjugated to 6-carboxyfluorescein succinimidyl ester

[000164] Also, Figure 9, provides binding isotherms for anti-PAP antibodies and PAP-fluorescein tracers. Shown are the N6-PAP-F8 tracer binding to Abs 1781 ○, 1810 ◇, and 3642 □, and C8-PAP-F14 tracer with the same antibodies: 1781 ●, 1810 ◆, 3642 ■. Antibodies were serially diluted two-fold in 50mM phosphate buffer (pH 7.4) containing 150 mM NaCl, 0.1 mg/mL BGG, and 1 nM tracer in a total volume of 100 uL in a 96-well plate or 12 ul in 384-well plates (results in the two plates are identical) and polarization values were read on the Tecan Ultra after one hour incubation at room temperature.

[000165] The binding properties of antibodies generated from the same immunogen in different animals were similar, but not identical. In this experiment, the 3642 antibody bound both tracers significantly tighter than 1781, and 1810 did not bind either tracer even at the highest concentration tested. The maximal polarization values for 3642 binding to N6-PAP-F8 and C8-PAP-F14 were 140 and 300mP, respectively; unbound tracer polarization was approximately 20mP, so the shift observed is more than adequate for use in an FPIA. To test whether binding is specific, competitive displacement by PAP was assessed.

[000166] Figure 10 shows PAP and PAPS competition curves for Ab 3642 and the same two tracers, PAP and PAPS. N6-PAP-6F8 tracer is represented by the open symbols: ○(PAP), □ (PAPS). C8-PAP-F14 tracer is represented with the closed symbols: ● (PAP), ■ (PAPS). PAP or PAPS was serially diluted two-fold in black mutiwell plates containing 50 mM phosphate buffer (pH 7.4), 150 mM NaCl, 0.1 mg/mL BGG, 1 nM C8-PAP-F14, and 1.5 uL purified Ab 3642 in a total volume of 12 µl or 1 nM N6-PAP-F8 and 0.5 uL 3642 Ab in a 20uL volume. Polarization values were read after one hour at room temperature.

[000167] The IC₅₀ for PAP with the C8-PAP-F14 tracer is 300nM, low enough to allow use of these reagents for monitoring SULT1E1 activity. Note that a much higher concentration of PAP (and PAPS) is required to compete off the tighter binding N6-PAP-F8 tracer. This may be because in this case the tracer has the same linker group as the

immunogen used to generate antibody, and a population of antibody is recognizing the linker, making the tracer more difficult to displace with free PAP. Figure 10 also shows that PAPS is less effective than PAP at displacing the tracers from Ab 3642.

[000168] Similar results were observed with the 1781 Ab as shown in Figure 11, which includes complete curves for PAP, PAPS and several similar molecules in competition experiments with the C8-PAP-F14 tracer. Figure 11 shows competition curves with two anti-PAP antibody/tracer combinations. Competitors were serially diluted two-fold in a black 384-well microtiter plate containing 50 mM phosphate buffer (pH 7.4), 150 mM NaCl, 0.1 mg/mL BGG, 1 nM Tracer C8-PAP-F14, and either 1.5 uL Ab 3642 (A) or 3 uL Ab 1781 (B) in a total volume of 12 uL. After one hour incubation at room temperature, the polarization values were read on a Tecan Ultra (Ex₄₈₅/Em₅₃₅). The mean and standard deviation of duplicates for all data sets are shown. The IC₅₀ values for PAP and PAPS with the 3641/tracer and 1781/tracer combinations were 0.3 uM PAP, 3.8 uM PAPS; and 0.3 uM PAP, 1.5 uM PAPS, respectively.

[000169] The 1781 and 3642 antibodies exhibited a 5- and 13-fold selectivity for PAP over PAPS respectively, and much higher selectivity for PAP over all of the other nucleotides tested. The cross reaction with PAPS is higher than expected given the lack of cross reaction with other adenine nucleotides. In this regard, it should be noted that most commercial PAPS preparations contain a significant fraction of PAP, but applicants purchased HPLC-purified preparations that were analyzed at greater than 95% purity, and took precautions in its storage and use to prevent hydrolysis. In any event, these results clearly show that applicants can generate antibodies that bind selectively to PAP in the presence of PAPS, which is a key feasibility issue for allowing sufficient signal:noise and dynamic range in the proposed SULT assay. An additional 10-fold increase in Ab selectivity for PAP over PAPS may be sufficient and is a very reasonable expectation using monoclonals rather than polyclonals.

[000170] The results observed with other antibodies and tracers tested were similar. The additional antibodies produced from the N6-aminohexyl-PAP-KLH and 8-azido-PAP-BSA immunogens bound many of the tracers and the Ab/tracer complexes could be displaced in all cases by unlabeled PAP, indicating that the interaction was specific. The 1810 antibodies showed very poor or no tracer binding and were not tested further. The polarization of the free tracers ranged from 15-40mP and increases observed upon Ab binding ranged from less than 100mP to almost 300mP. Interaction with some tracers was too weak to allow saturation with Ab, so maximal polarization changes were not always

observed. More than 2/3 of the tracers tested bound to both types of Ab, but none of the tracers synthesized from N6-carboxymethyl-PAP bound to antibodies generated from either immunogen.

The FPIA-based SULT assay

[000171] Though applicants will need to produce an antibody that differentiates between PAP and PAPS more effectively to develop a high quality assay, applicants were able to use the 3642 Ab and C8-PAP-F14 tracer to monitor detection of PAP produced in reactions containing the purified SULT1E1-cHis. In the initial experiment applicants sought to identify the optimal PAPS concentration for maximal signal:noise. SULT1E1-cHis was incubated with estradiol and varying concentrations of PAPS in the presence of pre-formed Ab-tracer complex; SULT1E1 was present at a level sufficient to rapidly drive the reactions to completion (Figure 12).

[000172] Figure 12 illustrates the effect of PAPS concentration on detection of enzymatically generated PAP. The assay mixture included 200 ng of SULT1E1-6xHis (□) or assay buffer (■) was added to wells containing 30 mM phosphate (pH 7.4), 7 mM DTT, 8 mM MgCl₂, 75 mM NaCl, 0.5 mg/mL BGG, 150 nM estradiol, 1 nM C8-PAP-F14 tracer, 12.5 uL Ab 3642, and varying concentrations of PAPS in a total assay volume of 100μL. The plate was incubated for 24 hour at 37°C and read in a Tecan Ultra (Ex₄₈₅/Em₅₃₅).

[000173] As in a typical FPIA, the tracer was used at a concentration well below the K_d and the Ab was adjusted to a concentration that resulted in approximately 85% of maximal tracer polarization in the absence of competitor. Figure 12 shows that there is a range of PAPS concentrations – from approximately 1 to 5mM – where the enzymatically produced PAP causes a decrease in tracer polarization of approximately 40mP (the difference between the open and solid squares in Figure 12).

[000174] That is, even though this antibody cross-reacts with PAPS significantly, it can be used in a competitive FPIA mode to detect PAP produced in a SULT reaction with saturating PAPS (K_m for PAPS with SULT1E1 is approximately 50nM) with a dynamic range of 40mP. Moreover, these were homogenous reactions, or single addition reactions, in which all of the reaction and detection components were added at the start of the reaction, which is the preferred approach for an HTS assay. Similar results are attained if the detection reagents are added after the enzymatic reaction is complete, thus the polarization signal is not affected by the enzymatic reaction (data not shown).

[000175] Described above is an embodiment of how one of the Ab/tracer pairs applicants produced was used for detection of PAP in SULT reactions that are allowed to proceed to completion before reading. However, a continuous assay is the most desirable format for HTS because it allows accurate enzyme rate determinations and precludes the need for a quench step. Figure 13 shows that the 3642 Ab and C8-PAP-F14 tracer can be used to continuously monitor SULT1E1 enzyme activity over time, allowing determination of enzyme rates with diverse substrates.

[000176] Figure 13 provides graphs of continuous FPIA-based detection of SULT activity with diverse substrates. acceptor substrates (200 nM) were added to wells containing 30 mM phosphate (pH 7.4), 7 mM DTT, 0.8 mM MgCl₂, 75 mM NaCl, 0.5 mg/mL BGG, 2 uM PAPS, 200 ng SULT1E1-cHis, 1 nM C8-PAP-F14 Tracer, and 12.5 uL Ab 3642 in a total volume of 100 uL. Control reactions (top trace in each graph) lacked C-His-SULT1E1 and contained all other reaction components. The plate was incubated at room temp and polarization read at 1 minute intervals.

[000177] The top trace in each graph is a control reaction lacking SULT1E1, thus polarization does not change significantly, whereas in the reactions containing enzyme it decreases with time. The experimental setup was very similar to what might be done in an HTS setting: antibody and tracer were put into the SULT reaction mix, dispensed into a 384 well plate containing different acceptor substrates, and the reactions were started by the addition of SULT1E1-cHis. The plates were then read at regular intervals and polarization values plotted as a function of time. In reactions with known SULT substrates, the polarization decreased over time relative to control reactions lacking enzyme; as may be expected as enzymatically produced PAP displaces the tracer from Ab. Note that there is no significant change in polarization when no acceptor substrate is present (graph f) or if SULT1E1 is absent (top trace in each graph), indicating that the PAPS molecule is sufficiently resistant to non-productive chemical or enzymatic hydrolysis.

[000178] These results show that a generic, fluorescence-based activity assay for sulfotransferases is technically feasible. In these experiments, the cross reaction of the 3642 Ab with PAPS contributes significant background (i.e., decrease in polarization), limiting the dynamic range of the assay, but enzymatic rates can still be obtained from the linear portions of the velocity curves. The bar graph in Figure 14 shows rates for each substrate calculated from linear portions of the velocity curves in Figure 13; though not a precise assay at this point, this rank-ordering of substrates does correlate inversely with

their published K_m values and with the substrate profile that applicants determined using purified SULT1E1 and the ^{35}S -PAPS radioassay (Figure 14). Accordingly, Figure 14 is a comparison of SULT1E1 acceptor substrate profiles determined using the FPIA-based assay and the ^{35}S -PAPS radioassay. Rates of FPIA-based reactions were calculated from linear portions of curves shown in Figure 13. It is noted that the ^{35}S radioassay data is provided in Table 4.

Acceptor substrates were used at 200nM (FPIA) or 400nM (^{35}S -radioassay).

[000179] Lastly, applicants used a known SULT inhibitor, DCNP, to show that the FPIA-based assay could be used to generate an inhibition curve (Figure 15). Specifically, Figure 15 is a graph showing inhibition of SULT1E1 by 2,6 Dichloro-4-nitrophenol (DCNP) measured with the FPIA-based assay. DCNP was serially diluted two-fold into wells in 46 μL of phosphate assay buffer (30 mM KPO_4 (pH 6.5), 0.5 mg/mL BGG, 15 mM DTT, 1.6 mM MgCl_2 , 4 μM PAPS), followed by 50 μL of a 2X Antibody/tracer mix (5 μL 3642 Ab/ 2 nM Tracer C8-PAP-F14), and 200 ng of SULT1E in a total volume of 100 μL . The plate was incubated at room temp for 30 min, and read on the Tecan Ultra. ΔmP values were calculated by subtracting the SULT1E reactions from the no SULT1E controls. All values represent the mean of replicates.

[000180] The response of the FPIA assay to DCNP was validated by comparison with the ^{35}S -radioassay (data not shown); the K_i values determined with the two assay methods were 7.8mM and 11mM, respectively. Thus applicants have demonstrated that the assay can be used for detection of substrates and inhibitors – both of the key intended HTS applications.

[000181] Taken together, these results clearly establish feasibility for producing all of the components of the proposed FPIA based SULT assay: purified recombinant SULTs, antibody that selectively recognizes PAP in the presence of PAPS, and fluorescent PAP tracers whose binding and competitive displacement from Ab can be detected by significant changes in polarization. In addition, applicants were able to demonstrate that the assay reagents could be used to detect SULT activity in a continuous mode, very similar to how the assay may be used in an HTS setting, that the response to different substrates is similar to the standard radioassay, and that the assay can be used for detection of an inhibitor.

[000182] Accordingly, it is envisioned that an antibody, suitably a monoclonal antibody with approximately 10-fold greater affinity and selectivity for PAP will be produced that will enable development of an assay with suitable dynamic range and signal:noise for commercial HTS applications.

Example 4. Assay Systems

[000183] Although, the methods described herein may be utilized in a variety of different assay systems, in its simplest form, the present assay system comprises an assay receptacle in which the assayed reaction is carried out, and a detector for detecting the results of that reaction. In preferred aspects, the assay receptacle is selected from a test tube, a well in a multiwell plate, or other similar reaction vessel. In such cases, the various reagents are introduced into the receptacle and typically assayed in the receptacle using an appropriate detection system, described above such as a fluorescence polarization detector. In addition to a receptacle, a flat surface such as glass or plastic could also be used and the reaction components spotted onto the surface in a defined array (such as a microarray).

[000184] Alternatively, and equally preferred is where the reaction receptacle comprises a fluidic channel, and preferably, a microfluidic channel. As used herein, the term microfluidic refers to a channel or other conduit that has at least one cross-sectional dimension in the range of from about 1 micron to about 500 micron. Examples of microfluidic devices useful for practicing the methods described herein include, e.g., those described in e.g., U.S. Pat. Nos. 5,942,443, 5,779,868, and International Patent Application No. WO 98/46438, the disclosures of which are incorporated herein by reference.

[000185] In accordance with the above-described methods, it is envisioned that an enzyme mediated coupling reaction between a first and second reactant may be carried out in channels of a microfluidic device. As such, by using a microfluidics platform, it may be possible to mimic the compartmentalization of a eukaryotic cell. This method could then be used to monitor the activity of group transfer reactions catalyzed by enzymes in a more native environment, in the context of other proteins and with cellular components that may affect enzymatic activity. Therefore, data on the activity of enzymes that catalyze group transfer reactions and the consequences of their inhibition can be obtained in a setting that will more accurately reflect an *in vivo* environment.

[000186] It is envisioned that these assay systems may be capable of screening test compounds that affect enzymatic reaction of interest. Optionally, devices used in accordance with the present invention are configured to operate in a high-throughput screening format, e.g., as described in U.S. Pat. No. 5,942,443. In particular, instead of delivering potential test compounds to the reaction zone from a reservoir integrated into the body of the device, such test compounds are introduced into the reaction zone via an external sampling pipettor or capillary that is attached to the body of the device and fluidly coupled to the reaction zone. Such pipettor systems are described in, e.g., U.S. Pat. No. 5,779,868 (fully incorporated by reference). The

sampling Pipettor is serially dipped into different sources of test compounds which are separately and serially brought into the reaction zone to ascertain their affect, if any, on the reaction of interest.

[000187] Movement of materials through the channels of these microfluidic channel networks is typically carried out using any of a variety of known techniques, including electrokinetic material movement (e.g., as described in U.S. Pat. No., 5,858,195 (fully incorporated by reference), pressure based flow, axial flow, gravity flow, or hybrids of any of these.

Example 5. Assay kit

[000188] Another embodiment of the invention is a kit for detecting and quantifying a Donor product of a group transfer reaction or a catalytic activity generating the donor-product of a group transfer reaction. The general equation for the group transfer reaction includes a donor- $X + \text{acceptor} \rightarrow \text{donor-product} + \text{acceptor} - X$, wherein the donor-product is detected by the general detection reaction: first complex + donor-product \rightarrow second complex + displaced detectable tag. In its most simplest form, the kit for an FPIA immunoassay may include a macromolecule (i.e., antibody or an inactivated enzyme) and a tracer (displaced) and optionally the specific group transfer enzyme of interest. It is noted that the macromolecule and the tracer may either be separate or incorporated into one solution vessel.

[000189] The kit may also include components such as, an activated donor, a detectable tag, acceptor substrates, inhibitors, buffers, cofactors, stabilizing agents, a set of instructions for using the kit, or packaging and any combination thereof. In addition, the kit may be formatted for multiplex detection by using more than one antibody/detectable tag pair where the detectable tags can be differentiated on the basis of the observables they produce. The immunoassay may be used to detect the donor product or the catalytic activity generating the donor product.

[000190] In practicing the invention, it is encompassed that the kit may be used for screening a library for a molecule or a set of molecules, capable of contacting an enzyme, wherein the enzyme generates the donor-product in a group transfer reaction. The library may include at least one of a plurality of chemical molecules, a plurality of nucleic acids, a plurality of peptides, or a plurality of proteins, and a combination thereof; wherein the screening is performed by a high-throughput screening technique using a multi-well plate or a microfluidic system.

[000191] It is further envisioned that the macromolecule in the kit includes at least one of an antibody, a polypeptide, a protein, a nucleic acid molecule, an inactivated enzyme, and a

combination thereof that is capable of contacting the donor-product with high affinity. It is further envisioned that the kit optionally include at least one of a sulfotransferase, a kinase or an UDP-glucuronosyl transferase, a methyl transferase, an acetyl transferase, a glutathione transferase, or an ADP-ribosyltransferase and combination thereof.

[000192] Although FPIA is a suitable mode of detection, also encompassed within the scope of the invention are kits designed to be used for detecting donor product or the catalytic activity generating the donor product through other means such as a homogenous assay, a homogeneous fluorescence intensity immunoassay, a homogeneous fluorescence lifetime immunoassay, a homogeneous fluorescence resonance energy transfer (FRET) immunoassay or a homogenous chemiluminescent immunoassay, or a non-homogenous assay such as enzyme-linked immunoassay (ELISA). In the case of a homogeneous fluorescence intensity immunoassay or a homogeneous fluorescence lifetime immunoassay, the kit could be composed of an antibody and fluorescent detectable tag where the intensity and/or lifetime of the detectable tag is different when it is bound to antibody and than it is free in solution. The difference in fluorescence; i.e., the assay signal, could be enhanced by modification of the antibody such that its interaction with the detectable tag results in a further change in its fluorescence properties; i.e., quenching, enhancement, or a change in the lifetime. In the case of a homogenous FRET immunoassay, the interaction of a first fluor associated with the detectable tag with a second fluor that is attached to the antibody - either directly or via associated binding molecules such as biotin and streptavidin - could result in the excitation of the second fluor (or the reverse), thereby generating a fluorescence emission at a wavelength different from that of the detectable tag. The second fluor could be a small organic molecule or a luminescent lanthanide probe. (It is noted that lanthanide emission is not fluorescence and is referred to as luminescence-based resonance energy transfer, or LRET). In the case of chemiluminescent detection, the detectable tag could be the donor product bound to one fragment of an enzyme used for chemiluminescent detection such as β -galactosidase. When the donor product-fragment-one complex is displaced from antibody by the donor product, it would then bind to fragment two of the enzyme, producing an intact, active enzyme that would be capable of producing a chemiluminescent signal with an appropriate substrate. In the case of an ELISA, the assay would not be homogenous, and would require donor product or antibody be immobilized to the surface of multiwell plates. A secondary antibody conjugated to a detection enzyme would also be included in this format.

[000193] All publications cited herein are hereby incorporated by reference in their entirety. In the case of conflict between the present disclosure and the incorporated publications, the present disclosure should control.

[000194] While the present invention has now been described and exemplified with some specificity, those skilled in the art will appreciate the various modifications, including variations, additions, and omissions that may be made in what has been described. Accordingly, it is intended that these modifications also be encompassed by the present invention and that the scope of the present invention be limited solely by the broadest interpretation that lawfully can be accorded the appended claims.